ONTOGENETIC STUDIES OF IMMUNITY AND TOLERANCE IN *XENOPUS LAEVIS* (DAUDIN)

by

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DEDICATED TO THE MEMORY OF OUR BELOVED DAUGHTER, AAMINAH

"To Allah do we belong and to Him do we return"

With Love.

DECLARATION

This work has not been accepted in substance for any other degree and is not concurrently being submitted in candidature for any other degree.

This is to certify that the work here submitted was carried out by the candidate himself. Due acknowledgement has been made of any assistance received.

Signed .....
(Candidate)

Signed .....
(Supervisor)

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ONTOGENETIC STUDIES OF IMMUNITY AND TOLERANCE IN XENOPUS LAEVIS (DAUDIN).

Abstract

Xenopus laevis injected with sheep erythrocytes (SRBC) at Stage 48 of Nieuwkoop and Faber (1967) showed no evidence of tolerance induction as a result of early exposure to antigen. These experiments showed that priming during the larval stages of development (at Stages 48 and 54 or at Stage 56) led to a positive anamnestic response when the animals were challenged after metamorphosis as toadlets. It was not, however, possible to demonstrate enhanced secondary responses within the larval period itself.

The effect of the alkylating agent, cyclophosphamide on the generation of immunological memory varies at different stages of development. In the larva, positive memory cells generated at the time of priming may be the population most affected by the drug, whereas in adults the evidence suggests that suppressor cells may have been eliminated by the cyclophosphamide treatment. Cyclophosphamide had no tolerogenic effect when administered with a primary injection of SRBC even at larval Stage 48.

It is concluded that, although transplantation tolerance to allografts has been demonstrated in Xenopus laevis, these free-living larvae are not vulnerable to tolerance induction by xenogeneic antigens. On the contrary, both HGG (human gamma globulin) and SRBC induce positive anamnesis in the larva which can be expressed post-metamorphosis. Tolerance was only observed in the present experiments to soluble antigen

(HGG) injected in high doses, such as could induce tolerance in the adult as well as in tadpoles.

In contrast to their ability to react to xenogeneic antigens with the induction of positive memory, larvae injected with live allogeneic cells (adult blood leucocytes) failed to produce any anamnestic response when challenged as toadlets. These cells, injected by various routes into larvae from Stage 47 to Stage 57 induced neither tolerance nor positive memory. The recipients responded in a primary manner both in mixed leucocyte reactions and to skin grafts.

- C H A P T E R O N E -

- C H A P T E R O N E -

GENERAL INTRODUCTION

The practical and theoretical importance of amphibians in the fields of developmental and comparative immunology has been emphhasised by several workers (Horton, 1969; Auerbach and Ruben, 1970; Du Pasquier, 1976b;Rimmer, 1976; Kobel and Du Pasquier, 1977; Katagiri, 1978; Edwards and Ruben, 1982; Gearing, 1983). One of the important features of amphibians is that they represent the transitional link between aquatic and terrestrial forms. They have been emphasised as a key step in the evolution of lymphoid tissues and of diverse immunoglobulin classes (Pollara et al., 1969). The study of their immunity thus helps in understanding how the immune system evolved and casts some light on the distinction between basic immune systems common to all vertebrate classes and adaptive systems specific to a particular class or species.

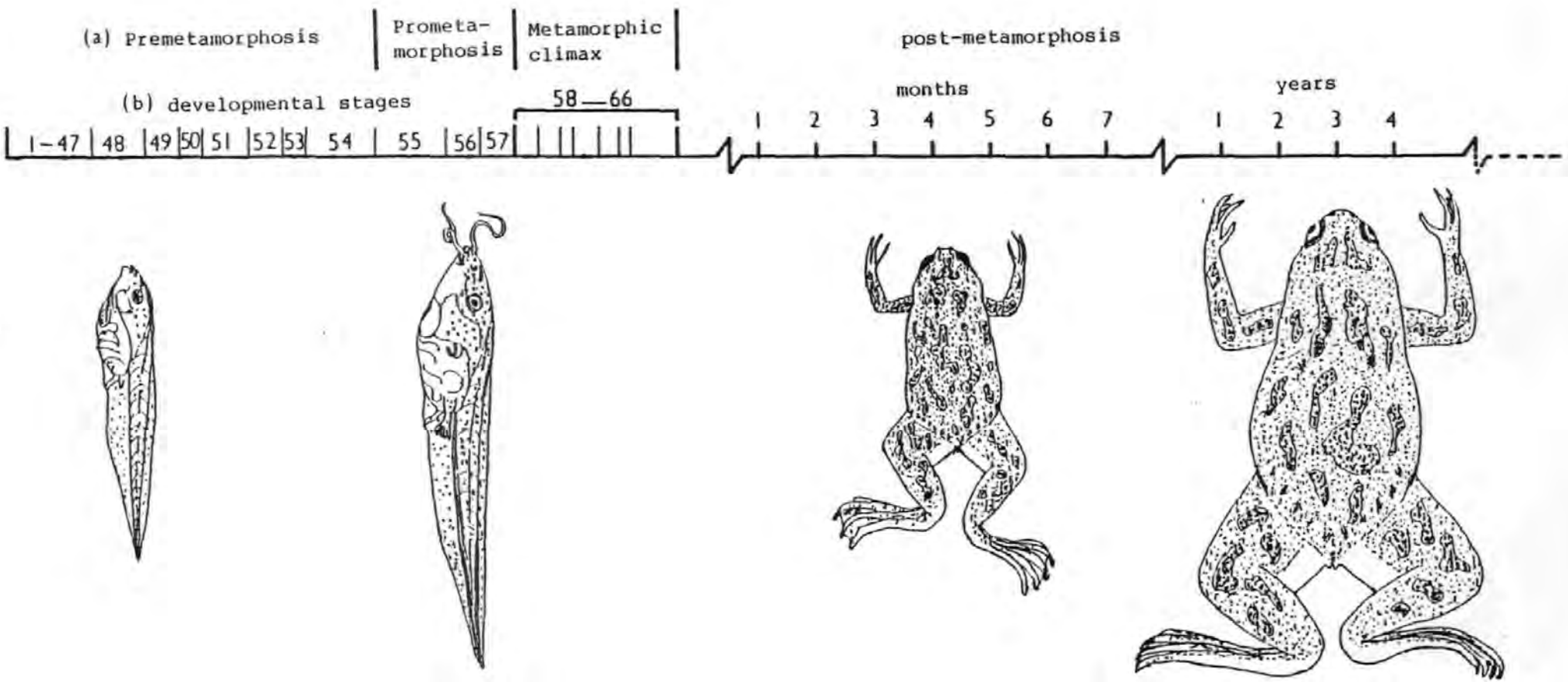
Amphibians have well defined lymphomyeloid organs, which include the thymus, spleen, kidneys and bone marrow and, in addition to these, anurans possess lymphomyeloid nodes and larval lymph glands (see Cooper, 1976; Manning and Turner, 1976; Manning, 1978, for comprehensive reviews). These organs seem to be more complex and show more specialization in anurans than in urodeles (Manning, 1978, 1980). Furthermore, urodeles seem to lack gut associated lymphoid nodules which can be found in anurans (Goldstine et al., 1975). Some of these structures appear for the first time, in evolutionary terms, in anurans. The spleen of anurans contains distinct white pulp and red pulp (Manning and Horton, 1969; Manning, 1981), and shows a complexity of structural

organisation which is not observed in urodeles or in fish. Both urodeles and anurans are capable of participating in a variety of immune responses, although in general terms anurans show stronger reactions (Edwards and Ruben, 1982). Urodeles produce only one type of antibody (the heavy molecular weight immunoglobulin IgM) in response to antigenic stimulation (Marchalonis, 1971; Du Pasquier, 1973). Urodeles display the presence of T and B cell co-operative function in their response to hapten-carrier conjugates (Ruben et al., 1973; Tahan and Jurd, 1981). This response indicates the presence of helper-function in this order, which also shows memory response to some antigens (Charlemagne and Tournefier, 1977; Charlemagne, 1982). Urodeles were also shown to reject allografts chronically when compared with the more advanced anurans (Cohen, 1969, 1970, 1971; Cooper, 1976; Tahan and Jurd, 1983). Anurans have low (7S) as well as high molecular weight (19S) antibodies both as tadpoles and as adults (Hadji-Azimi, 1971, 1979; Du Pasquier, 1973, 1976, 1982; Cooper, 1976), complement (Ohnishi et al., 1981; McKnight, 1982) and lymphokine (Gearing, 1983) systems. They also have helper (Ruben et al., 1977, 1980a, 1980b) and suppressor (Du Pasquier and Bernard, 1980; Du Pasquier, 1982) mechanisms as well as an efficient memory to various antigens (Koppenheffer and Inchalik, 1979; Wright and Cooper, 1980; Du Pasquier, 1982). Recently Jurd and Doritis (1977) found antibody mediated cytotoxicity reactivity in the spleen cells of Xenopus laevis. These immunological characteristics of anuran amphibians make them suitable for the study of memory mechanisms.

The information recorded in the literature about the immune system and immunity in anurans is reviewed by: Cooper, 1973, 1976; Du Pasquier, 1973, 1976, 1982; Manning and Turner, 1976; Manning, 1978, 1979, 1980, 1981; Cohen and Turpin, 1980; Edwards and Ruben, 1982. First it must be

noted that anurans enjoy a special interest for being phylogenetically the most advanced amphibians and for encompassing two contrasting developmental stages during their lives; they begin life as an entirely aquatic larva (tadpoles) and later convert into a tailless, partially terrestrial adult. This conversion, known as metamorphosis, takes a variable length of time depending firstly, on the species and secondly, on various environmental conditions such as temperature, nutrients and population density. The variations within one spawning of one species results in the co-existence of animals at various stages of development. The onset of metamorphosis is a gradual process, some changes take place much earlier or later than others. This makes it difficult to determine the start of the metamorphic period. Nevertheless, it is possible to notice a period when the metamorphic process takes an accelerated step at the end of which a miniature adult emerges; this is termed a metamorphic climax (Dodd and Dodd, 1976). In Xenopus laevis it commences at Stage 58 (of Nieuwkoop and Faber, 1967) (44 days old at 23^{o}C) and ends by the beginning of Stage 66 (58 days old) (Dodd and Dodd, 1976) (Figure 1). Under complex hormonal influence metamorphosis results in morphological, biochemical, physiological and behavioural changes switching larval aquatic systems to adult terrestrial ones (Dodd and Dodd, 1976; White and Nicoll, 1981). Some are obvious like the loss of the tail and the adult development of the skin and limbs, others are more internal like the loss of the gills and development of the lungs. The pronephros (in the kidney) degenerates gradually from Stage 53 (Nieuwkoop and Faber, 1967) until the end of metamorphosis when only the mesonephros constitutes the kidney (Fox, 1981). The gut shortens considerably and there is striking cellular degeneration and regrowth in different regions (Bonneville and Weinstock, 1970; Houdry and Dauca, 1977; Sumiya and Horiuchi, 1980; Fox, 1981), the luminal cilia are

Figure 1 - Diagramatic representation of the normal development of Xenopus laevis (Daudin) at 22°-24°C.



(a) Classification from Dodd and Dodd, 1976.

(b) Classification from Nieuwkoop and Faber, 1967.

replaced by microvilli to provide the large surface area for the absorption of digested food (Fox et al., 1972). The pancreas is also reported to undergo various structural and morphological changes (Race et al., 1966 for Rana pipiens; Leone et al., 1976 for Xenopus laevis). Almost every larval organ undergoes some degree of morphological, biochemical, physiological or functional modification (see Sedra and Michael, 1957; Dodd and Dodd, 1976; Gilbert and Frieden, 1981; Wald, 1981; Just et al., 1981), and there are distinct changes in the erythrocytes and their haemoglobin (Maclean and Jurd, 1971; Just et al., 1977; Cardellini and Sala, 1979; Just et al., 1980; Broyles, 1981). The difference in molecular structure between larval and adult haemoglobin underline differences in some structural genes (Broyles, 1981), larval erythrocytes are replaced by adult ones concomitently with a change in the site of their production (Broyles, 1981). Larval mesonephric kidney and liver produce larval erythrocytes. These are replaced by production in the liver alone shortly after metamorphosis and by production in the spleen and bone marrow in the adult (Broyles, 1981). It is thought that larval erythrocytes (RBC) are actively eliminated during the metamorphic climax while being replaced by new adult RBC populations (Just et al., 1980). In fact, larval RBC were observed to be undergoing intense phagocytosis in the liver of Xenopus laevis tadpoles subject to induced metamorphosis (Kistler and Weber, 1975). Macrophages are in fact thought to be actively involved in the process of resorbing the various degenerating organs such as the kidney and tail of metamorphosing animals (Lehman, 1953; Dodd and Dodd, 1976; Fox, 1981).

In the adult anuran, the mature fully differentiated thymus

consists of a medullary mass of cells encased in a histologically denser cortex (Sterba, 1950; Cooper, 1967; Manning, 1981). The architectural structure of these two "regions" consists of an inter-woven skeleton of epithelial cells the iterstices of which are filled with lymphocytes. It is now believed that these are derived from an extra thymic source (Tompkins et al., 1979; Volpe et al., 1979; Turpin, 1980; Tompkins et al., 1980; Tochinai, 1980; Volpe et al., 1981).

The thymus in anurans is thought to contain mainly T cells, nevertheless some B cell activity has been reported to occur in this organ. This is demonstrated by the presence of antibody producing cells (Moticka et al., 1973) and rosette forming cells (RFC) which appear in the thymus in response to foreign erythrocytes (Kidder et al., 1973; Minagawa et al., 1975). This is further supported by some experimental evidence of B cell subsets (responsive to Ficoll) possibly spawned by the thymus (Horton et al., 1979). On the other hand, several workers (Horton and Horton, 1975; Manning and Collie, 1975; Rimmer and Horton, 1977) found that toads that had been thymectomized at a late larval stage and grafted with allogeneic skin reject their grafts in a normal first set rate. This is thought to indicate either the presence of allo-reactive clones of cells resident outside of the thymus of the older larva (Horton and Horton, 1975) or the occurrence of a non-thymic differentiation pathway of at least some T cell functions (Manning and Collie, 1975). Ontogenetically the thymus is the first lymphoid organ to develop in Xenopus tadpoles appearing as an anlage at Stage 42 (possibly Stage 41 (Kaye and Tompkins, 1983)), budding inwards from the pharyngeal epithelium (Manning and Horton, 1969). It finally detaches at Stage 47 while still increasing in size and by Stage 49 it doubles in volume and contains small lymphocytes in two distinct zones, the medulla

and the cortex (Manning and Horton, 1969). This differentiation is also reported in other species of anurans: Rana sylvatica (Fabrizzio and Charipper, 1941); Rana catesbeiana (Hildemann and Hass, 1962; Cooper, 1967) and Rana pipiens (Horton, 1971b; Curtis and Volpe, 1971) (see Du Pasquier, 1973). By Stage 51 the thymus is fully differentiated and remains as such until the beginning of metamorphosis (Stage 58), then its lymphocytic content begins to decrease, reaching a low point at the end of metamorphosis (Stage 66) after which it increases, only to decrease again during adulthood (Du Pasquier, 1976). Within the last decade it has become apparent that, in Xenopus at least, the early larval thymus plays a major role in the maturation of some immune responses consequently considered to be thymus dependent (TD). Thus adults that have been thymectomized at a larval stage may show little, if any, TD responses. These include antibody production against foreign erythrocytes (Turner and Manning, 1974; Tochinai and Katagiri, 1975; Horton et al., 1976), skin graft rejection (in Xenopus, Horton and Manning, 1972; Horton and Horton, 1975; Manning and Collie, 1975; Rimmer and Horton, 1977; Tompkins and Kaye, 1981; Kaye and Tompkins, 1983, in Rana catesbeiana, Cooper and Hildemann, 1969 (larvae); in Rana pipiens, Curtis and Volpe, 1971), lymphocyte depletion from the thymus dependent regions of lymphoid organs (Manning, 1971; Horton and Manning, 1974; Tochinai, 1976), depletion of surface immunoglobulin positive lymphocytes in the spleen (Weiss et al., 1973), and in vitro proliferative response to T cell mitogens (Phytohaemagglutinin (PHA) and Concavalin A (Con A)) (Donnelly et al., 1977; Manning et al., 1977; Green and Cohen, 1979; Horton and Sherif, 1977; Rollins-Smith and Cohen, 1982) and to alloantigens in mixed leucocyte cultures (MLC) (Du Pasquier and Horton, 1976).

Unlike the thymus which produces T-cells, the adult lymph nodes

and larval lymph glands are considered to be secondary lymphoid organs. They are possessed by most anurans but adult lymph nodes are absent from the primitive anuran Xenopus laevis (Manning and Horton, 1969) while larval lymph glands were recently found to be absent in Rana temporaria (Plytycz and Bigaj, 1982). In Ranid and Bufid anurans the lymph nodes and lymph glands consist of accumulations of lymphoid tissue surrounding several connected blood sinusoids (Manning, 1981; see also Cooper, 1968, 1976; Cooper et al., 1975). The function of these structures in adults seems to be for filtering blood rather than lymph (Baculi and Cooper, 1967) while in tadpoles of Rana pipiens the larval lymph glands filter both blood and lymph (Horton, 1971b; Cooper, 1973, 1976; Manning, 1981). The adult lymph nodes play a major role in antigen trapping (Kent et al., 1964; Diener and Nossal, 1966; Diener and Marchalonis, 1970; Manning, 1981). In tadpoles, however, it is not certain what role the lymph glands play in the immune response but they are predominantly lymphoid (Cooper, 1967). They were found to contain a germinal centre (Cooper et al., 1971) and their removal depressed antibody production to T dependent antigens but did not affect the ability to reject allografts (Cooper, 1968). Cooper et al., (1975) reported the possible presence of stem cells that can differentiate into B cells and T cells in the lymph glands of bull-frogs (Rana catesbeiana) larvae; autologous lymph gland cells restore to some extent, both antibody production and allograft rejection facilities in bilaterally lymph glandectomised and total body irradiated tadpoles (Cooper et al., 1975). These structures first appear at an early larval stage (Stage 2 (9-12 days old)) in Rana pipiens at 18 - 21^{0}C (Horton, 1971b) equivalent to Stages 47-49 in Xenopus laevis (Nieuwkoop and Faber, 1967). By day 12 they begin to assume their typical structure but it is not until 20-27 days post-fertilization that they have differentiated lymphocytically and contain lymphocytes of various sizes, reticulo-endothelial cells and blast cells in the cords and myeloid cells

in the sinusoids (Cooper, 1976). These glands continue to grow in size until metamorphosis when they disappear (Riviere and Cooper, 1973; Cooper, 1967, 1976). Adult lymph nodes, however, develop during metamorphosis (Horton, 1971a). They have a similar structure to that of the larval lymph glands (Cooper, 1976).

The spleen of amphibians is another of the secondary lymphoid organs. The mature, fully differentiated spleen in anurans is a capsule of connective tissues holding a mass of 'red pulp' cells intermingled with a cluster of dense lymphocytic accumulations (white pulp). The white pulp cluster is connected by the branching central arteriole, and separated from the red pulp by a double layer of epithelial cells (boundary layer) (Manning and Horton, 1969; Manning, 1981). Identification of the thymus-dependent areas of the spleen have been attempted by several workers during the last 12 years (Manning, 1971; Weiss et al., 1973; Horton and Manning, 1974b; Manning et al., 1977; Tochinai, 1976; Donnelly et al., 1977; Horton and Sherif, 1977; Green and Cohen, 1979; Horton et al., 1980; Obara, 1982; Rollins-Smith and Cohen, 1982). Thymus-dependent areas of the spleen were identified as the perifollicular lymphocytic areas in the red pulp surrounding the boundary layer (Manning, 1971). Radioactively labelled thymocytes injected into normal and thymectomized Xenopus toads were almost entirely detected in the red pulp area, more so in thymectomized animals. Injection of labelled splenocytes or label (tritiated thymidine) alone showed higher concentrations of labelled cells in the white pulp or an even distribution respectively (Obara, 1982). Splenic T dependent cells were functionally identified by their responsiveness to T cell mitogens, PHA and Con A, in Xenopus (Du Pasquier and Horton, 1976; Manning et al., 1977; Horton et al., 1980; Williams and Horton,

1981). The development of the spleen in Xenopus laevis begins with the appearance of its anlage at Stage 45 (of Nieuwkoop and Faber, 1967) and its lymphoid differentiation becomes evident at Stage 50 when small lymphocytes can be seen in the white pulp (Manning and Horton, 1969). The differentiation of the white and red pulps can be seen at Stage 47 but it is not until Stage 50 when the boundary layer between these becomes apparent (Manning and Horton, 1969); from Stage 50 onwards, the spleen increases in size and number of white pulp nodules until the beginning of the metamorphosis climax at Stage 57 when its lymphocytic content decreases. This fall in cell numbers continues until the end of metamorphosis then the numbers steadily increase (Du Pasquier and Weiss, 1973). In another anuran Rana pipiens development of the spleen commences with the apparent formation of the anlage at 7-9 days of age at 18 - 21^{0}C. The spleen of 20-27 days old tadpoles bears some scattered small lymphocytes and later at 30-35 days of age the presence of lymphocytes of all sizes marks the lymphoid maturation of the spleen. The differentiation into red and white pulps becomes noticeable in 68-80 day old tadpoles (Horton, 1971b).

Splenocytes of normal Xenopus laevis have the ability to respond in vitro to stimulation by antigen (Auerbach and Ruben, 1970) and by allogeneic cells (Weiss and Du Pasquier, 1973; Bernard et al., 1979). In the intact animal, the spleen of immunized Xenopus shows an increase of proliferating cells (Turner and Manning, 1973; Horton et al., 1977) which is believed to precede antibody production by a few days (Sima et al., 1981). Turner and Manning (1973) have also reported an increase in the weight of spleens from immunized animals with an increase in the number of pyroninophilic cells in the white pulp.

The role of the spleen as an antigen trapping site playing an important role in antibody production was emphasized by Turner (1973) and Collie and Turner (1975). They found that splenectomized adult Xenopus laevis failed to produce antibodies to threshold low doses of antigen. They suggest that the spleen acts as an antigen concentration site. This is essential when the antigen is administered in low doses, whereas when high doses of antigen are given there is sufficient antigen concentration to have an immunizing effect in the absence of the spleen because antigen comes into contact with antibody producing cells in sinusoidal organs such as the kidneys, where the circulation is sluggish.

Other lymphoid organs in Xenopus laevis such as the lymphocytic component of the kidney and the ventral and dorsal cavity bodies develop at Stages 48 and 49 respectively, while the liver contains lymphomyeloid tissue for the first time at Stage 49 (Manning and Horton, 1969; Tochinai, 1975b). All three types of organ attain their lymphoid differentiation at Stage 50, when small lymphocytes can be seen in their tissues. These organs continue to grow in size throughout the larval period. The ventral and dorsal cavity bodies which are in Xenopus laevis located in the branchial region disappear with the region during the metamorphosis climax (Manning and Horton, 1969).

In Rana pipiens the lymphomyeloid kidney differentiates at approximately 68 days of age at 18 - 21^{0}C, the ventral cavity bodies after 19 days of age (Horton, 1971a,b) and the liver contains cells identified as B cells at 12 days of age and plasma cells at 24-28 days (Zettergren, 1982). The ventral cavity bodies of Rana disappear during metamorphosis as do those in Xenopus. The precise role of these organs

in the immune response is not certain but B cell generation in the liver is suspected in both Xenopus (Zettergren, 1977) and Rana (Zettergren, 1982). Furthermore, lipopolysaccharide of E. Coli (LPS) (B cell mitogen) reactive cells were detected in the liver of Xenopus (in tadpoles, DiMarzo and Cohen; reported in Cohen and Turpen 1980 and in adults, Green and Cohen, 1979).

The immunologically functional bone marrow is present in adult anurans (Ramirez and Cooper, 1980; Cooper et al., 1980) but not their early tadpoles since tadpoles do not have a bony skeleton; ossification in Xenopus starts at Stage 56 (with the beginning of the metamorphic climax period) (Sedra and Michael, 1957). The newly formed bone marrow of 80 day old leopard frog tadpoles (Rana pipiens), however, has been reported to contain B cells identifiable by their surface immunoglobulins (Zettergren, 1982). Recently, adult bone marrow of Rana pipiens was found to be capable of maintaining the viability of lethally irradiated frogs (Ramirez et al., 1983). Ramirez et al. (1983) also found that bone marrow was efficient in maintaining the responsiveness to T cell mitogens (for example, Con A) and the production of RFC in response to sheep erythrocytes (SRBC) following irradiation.

There has been considerable interest in the lymphoid cells of lower vertebrates which bear membrane-associated immunoglobulin on their surface. It is generally thought that immunoglobulin (Ig) synthesis commences concomitantly with the appearance of lymphocytes expressing Ig molecules on their surfaces. In Xenopus laevis recently, two day old embryos at the hatching stage (Stage 35 of Nieuwkoop and Faber, 1967) were found to contain extra cellular 19S high molecular

weight immunoglobulin (IgM) with properties similar to adult IgM (Moyer et al., 1977; Leverone et al., 1979; Bruning et al., 1981). Bruning et al., (1981) have also isolated lymphocyte-like cells from these embryos, which they suggest are responsible for the production of the IgM; these cells contain messenger RNA (m RNA) coding for IgM heavy chain. These authors have also reported detecting surface immunoglobulin positive lymphocytes in embryos at Stage 35 (2 day old) and subsequent stages up to the end of their studies, i.e. up to Stage 46 (4-5 days old). Studies by other workers detected surface immunoglobulin positive (SIg+) cells throughout the larval period in Xenopus laevis and beyond that into their adulthood (Du Pasquier, 1973; Jurd and Stevenson, 1976; for review see Du Pasquier, 1982). These observations were made for the thymus, spleen and peripheral blood lymphocytes. The numbers of these cells (SIg+) in the thymus were reported to increase during the larval period from Stage 46 until they reach a maximal plateau at Stage 48 which extends until two to three weeks post-metamorphosis when they decrease to reach very low levels (1% of the total lymphocyte number in the thymus) (Du Pasquier et al., 1972; Du Pasquier and Weiss, 1973; Jurd and Stevenson, 1976). These authors have also reported that during the metamorphic climax the numbers of these SIg+ cells drop below the level of their plateau then reattain it once again before they decrease as mentioned above. SIg+ lymphocytes in the peripheral blood and spleen, however, remain more or less at the same level throughout the metamorphic climax. The adult kidneys and gut associated lymphoid tissues have also been found to house SIg+ cells (Jurd and Stevenson, 1976; Jurd and Manning, 1980). In studies on the Ig class of cell surface immunoglobulins, both IgM and low molecular weight immunoglobulin (LMW Ig) were identified (Jurd and Stevenson, 1976; Jurd and Manning, 1980; Hadji-Azimi and Schwager,

1980, 1982), the majority of cells bearing SIg of the IgM class. While some workers reported the presence of SIg+ lymphocytes in larval Xenopus thymuses (Du Pasquier et al., 1972; Du Pasquier and Weiss, 1973; Jurd and Stevenson, 1976), others have reported the contrary (Hadji-Azimi and Schwager, 1980, 1981, 1982). Hadji-Azimi and her colleague have obtained results indicating that there are common cross reacting carbohydrate determinants on both the surfaces of thymocytes and SIgM in these animals (Hadji-Azimi and Schwager, 1980).

In spite of the early appearance of serum immunoglobulin and SIg+ lymphocytes (considered to be B cells by the earlier workers according to higher vertebrate criteria; Cooper et al., 1972; Owen, 1972), specific humoral responses, at least in the studied anuran species, commence later (Du Pasquier, 1982). As early as the first appearance of small lymphocytes in the spleen, Xenopus tadpoles at Stage 49 produce specific antigen binding cells detectable in their spleens after immunization with sheep erythrocytes (SRBC) (Kidder et al., 1973). Whether in Kidder's experiments (Kidder et al., 1973) tadpoles at the earlier stage of Stage 48 are completely unable to respond to immunogenic stimulation was not clear, since their experiments did not extend beyond detecting the primary responsiveness. Jurd et al. (1975), however, reported that tadpoles implanted at this stage with alum precipitated human immunoglobulin light chain, although unable to give an antibody response, produced what may be considered as a secondary reaction when challenged at later stages (Stages 53-56), i.e. Stage 48 immunization had primed the immune system. Whether the unresponsiveness reported by Kidder and his colleagues (1973) was due to a state of tolerance to the antigen (sheep erythrocytes, SRBC) or

was merely a condition of unresponsiveness is yet to be investigated.

Among the other characteristics of the immune response in anuran larvae is the cell to cell collaboration in response to hapten-carrier systems (Haimovich and Du Pasquier, 1973; Du Pasquier, 1973; Du Pasquier and Haimovich, 1974,1976; Ruben et al., 1980b). At the cellular level, these responses were detected in Xenopus tadpoles at Stage 51 and from then onwards through the metamorphic period into adulthood (Ruben et al., 1980b). In these experiments helper mechanisms seem to increase during the climactic period of metamorphosis, which led the authors (Ruben et al., 1980b) to suggest the retardation of regulatory suppression during that period. The co-operative system depends to some extent on the carrier used to present the hapten; thymus dependent carriers such as sheep erythrocytes or Ficoll required the presence of thymus helper cells, whereas a thymus independent carrier (LPS) did not (Horton et al., 1979). Another example of cell to cell co-operation comes from the response to heterologous erythrocytes (foreign RBC) (Ruben et al., 1977; Edwards and Rosene, 1979). These experiments demonstrated the prerequisite presence of T-helper cells for a humoral response by splenic effector cells to be detected.

Anuran tadpoles are also capable of rejecting allografts, the ontogeny of which was studied in Xenopus laevis by Horton (1969). He found that Xenopus tadpoles were able to launch a population of lymphocytes which infiltrated and accumulated underneath the skin graft. This was considered an alloreaction although actual rejection of the grafts was not reported. This, the author found, occured in concomitance with the lymphocytic differentiation of the thymus at Stage 49 (Horton and Manning, 1972).

Allograft rejection in Xenopus laevis is apparently thymus-dependent in the sense that removal of the thymus from the animal at an early larval stage abrogates this responsiveness (Horton and Manning, 1972; Horton and Horton, 1975; Tochinai and Katagiri, 1975; Rimmer and Horton, 1977; Manning and Collie, 1977; Kaye and Tompkins, 1983). The earliest stage at which thymectomy is effective was found to be Stage 41 (Kaye and Tompkins, 1983), whereas thymectomy was found ineffective in abrogating allograft rejection when done after Stage 51 (Kaye and Tompkins, 1983).

During their metamorphosis Xenopus tadpoles were reported to be unable to reject allografts from donors differing by their minor histocompatibility antigens or by one major histocompatibility haplotype (Chardonnens and Du Pasquier, 1973; Du Pasquier and Chardonnens, 1975; Cohen et al., 1980; Barlow et al., 1981). Such operations were also found to lead to a state of specific tolerance and thought to be regulated by a population of suppressor cells (Du Pasquier and Bernard, 1980; Du Pasquier, 1982). Other workers have reported that such transplantation tolerance may be induced by this method throughout the larval period, depending on the size of the graft and histocompatibility differences between graft donors and hosts (DiMarzo and Cohen, 1979; Cohen et al., 1980).

Alloreactivity in anurans can also be demonstrated in mixed leucocyte cultures (MLC) (Weiss and Du Pasquier, 1973; Du Pasquier and Weiss, 1973). For technical reasons the earliest stage of development at which alloreactivity of Xenopus tadpoles in MLC was detected was Stage 55 (Du Pasquier and Weiss, 1973). Pre-immunization by skin grafting was found, to a certain extent, to increase the reactivity of

graft host cells against graft donor cells in MLC (Barlow and Cohen, 1981). Another *in vitro* reactivity studied in *Xenopus* (Manning et al., 1977; Donnelly et al., 1977; Horton and Sherif, 1977; Williams and Horton, 1981) as well as in *Rana pipiens* (Goldstine et al., 1975a; Goldstine, 1977; Wright and Cooper, 1978; Rollins-Smith and Cohen, 1982) and *Bufo marinus* (Goldshein and Cohen, 1972) is responsiveness to T and B cell mitogens. These studies revealed that in these anurans there are two main populations of cells; one responsive to mammalian T (thymus) cell mitogens such as PHA and Con A, found in both the thymus and spleen and sensitive to early thymectomy, the other responsive to mammalian B (bursa or bone marrow) cell mitogens such as LPS and purified protein derivative of tuberculin (PPD), resistant to early thymectomy and mainly found in the spleen. Lymphocytes responsive to the B cell mitogen LPS were detected in the thymuses of *Xenopus* toads and were removable by absorbtion through a nylon wool column. There were thought to be B cells, possibly spawned by the thymus (Williams and Horton, 1981), since the latter method is used to remove B cells from cell suspensions in *Xenopus laevis* (Bernard et al., 1979) and in *Rana pipiens* (Klempau and Cooper, 1983).

Tadpoles of both *Xenopus laevis* and *Rana pipiens* possess mitogen responsive lymphocytes; their cells were found to respond to LPS (Horton et al., 1980; Williams and Horton, 1981; Rollins-Smith and Cohen, 1982), PHA (Rollins-Smith and Cohen, 1982) and Con A (Williams and Horton, 1981). These responses were detectable throughout the larval development of *Xenopus laevis* starting from Stage 56 and seem to decrease during metamorphosis (Horton et al., 1980; Williams and Horton, 1981).

Adult un-immunized anurans produce both 19S and 7S immunoglobulins in their sera (Rana esculenta and Rana temporaria, Trnka and Franek, 1960; Rana catesbeiana, Marchalonis and Edelman, 1966; Marchalonis, 1971; Hadji-Azimi, 1971; Rana esculenta, Du Pasquier, 1969; Bufo marinus, Marchalonis and Germain, 1980; Xenopus laevis, Lykakis, 1969; Hadji-Azimi, 1971; Turner and Manning, 1974; Jurd and Manning, 1980). There is, however, some doubt as to whether un-immunized tadpoles produce the low molecular weight (7S) class of immunoglobulin (LWM Ig) as well as the high molecular weight (19S) immunoglobulin (IgM). Marchalonis (1971) has reported that the sera of Rana pipiens tadpoles contained only IgM while other workers have later detected LWM Ig in the tadpole sera of Rana pipiens and Rana clamitans (Geczy et al., 1973), Rana catesbeiana (Geczy et al., 1973; Du Pasquier, 1973; Du Pasquier, 1976) and Rana temporaria, Rana esculenta and Xenopus laevis (Du Pasquier, 1973). But what seems to be a point of agreement is that anuran tadpoles may not be able to produce LMW Ig antibodies in response to antigenic stimulation very easily. This arouses the interest in investigating whether such tadpoles have the potential to respond anamnestically to antigenic challenge. There is some indication that they may not be able to do so; Moticka et al. (1973) reported on the inability of bullfrog tadpoles to produce a true anamnestic response to SRBC. Such tadpoles, however, were reported to produce LMW Ig in response to a second immunization with the bacteriophage f2 (Green and Steiner, 1976) although this often required more than one injection of the antigen (Geczy et al., 1973). Other workers have also reported the production of LMW Ig, albeit in low quantities, by Xenopus tadpoles in response to implants of alum precipitated human immunoglobulin light chain (Jurd and Stevenson, 1976).

It appears that the class of antibody produced after primary immunization depends on the type of antigen used for immunization (Hadji-Azimi and Schwager, 1980). This is true for adult anurans and may be true for their larvae. Salmonella, either killed whole bacteria or their flagellae, induce only IgM production in Bufo marinus (Legler et al., 1969; Marchalonis and Germain, 1980) and in Xenopus laevis and Rana catesbeiana (Du Pasquier et al., 1972). Whereas Limulus hemocyanin, dinitrophenyl conjugated keyhole limpet hemocyanin (DNP-KLH), human gamma globulin (HGG) and bovine gamma globulin (BGG) provoke both IgM and LMW Ig in Xenopus laevis (Lykakis, 1969; Marchalonis et al., 1970; Turner and Manning, 1974; Du Pasquier and Haimovich, 1976). Bovine serum albumin (BSA) and the bacteriophage f2 provoked the production of both IgM and LMW Ig in Rana catesbeiana and Bufo marinus (Marchalonis and Edelman, 1966; Legler et al., 1969; Lin et al., 1971; Geczy et al., 1973; Green and Steiner, 1976; Marchalonis and Germain, 1980). Only IgM was found to be produced by Xenopus laevis in response to LPS and SRBC (Turner and Manning, 1974; Collie et al., 1975), while Bufo marinus produced both IgM and LMWIg in response to horse RBC (HRBC) (Marchalonis and Germain, 1980). Secondary immunization with some antigens in adult anurans increases LMWIg production (Bufo marinus, Lin et al., 1971; Marchalonis and Germain, 1980; Xenopus laevis, Mancini et al., 1965; Hadji-Azimi, 1971, 1979). There may be a correlation between the production of LMWIg in response to an antigen and the production of an anamnestic response. Anamnesis was not produced in Bufo marinus for immunization with Salmonella adelaide which did not provoke LMWIg production (Legler et al., 1969; Marchalonis and Germain, 1980).

In spite of the wealth of knowledge on anuran immunity and apart

from work involving transplantation experiments (see above), there have been relatively few studies on memory function during ontogeny. Immunological memory (anamnesis) in general terms is characterized by some degree of an exaggerated response directed, usually specifically, towards either a faster and more efficient elimination of the antigen (positive anamnesis) or towards the acceptance and endurance of the antigenic presence (tolerance). It may involve both T and B cells, both cell types can carry immunological memory to a T-dependent antigen (requiring T-helper memory cells) and can co-operate in the production of a humoral response (for review see: Celada, 1971; Miller, 1973; also see Manning, 1980). In this context any modification of an immune response to antigenic challenge conferred by earlier exposure to the same antigen may be termed as specific immunological memory. It is fairly easy to induce positive memory, tolerance on the other hand may be subject to many restricting conditions some of which depend on the host's condition, while the others depend on the antigen itself.

In anuran amphibians there have been relatively few studies on the induction of either aspect of memory. This is the interest of the present study using the amphibian anuran species Xenopus laevis (Daudin) of the Pipidae family, since it is one of the best known anuran species. Xenopus laevis is a particularly suitable animal with which to study the ontogeny of the mechanisms involved in both immunity and tolerance. The animals are easily maintained under laboratory conditions since they accept inert food at all developmental stages and can be bred at any time during the year. In ontogenetic studies the particular suitability of Xenopus lies in their easily manipulatable, transparent and free-living aquatic larvae (tadpoles). These tadpoles are spawned after each successful mating in large numbers. In addition to the above

points the histogenesis of the lymphoid organs of Xenopus laevis has been extensively described (Manning and Horton, 1969). Nieuwkoop and Faber (1967) described the morphology of the normal developmental stages of Xenopus from the onset of fertilization. Analytical and molecular aspects of their development have also been described (Deuchar, 1976). In addition, their arterial and venous circulations have been extensively described and illustrated by Millard (1941).

One of the aspects of immunological memory relating to the concept of self-recognition is the mechanism(s) of tolerance. In the present study three different types of antigen were used, a non-living cellular antigen (sheep erythrocytes), live allogeneic cells and a soluble foreign protein (human gammaglobulin) to determine whether or not they can induce tolerance in Xenopus laevis and to investigate some of the mechanisms involved. More specifically the present study attempts to answer the following questions:-

1) Can Xenopus tadpoles respond in an anamnestic fashion to antigenic challenge?

2) How are immunological memory responses affected by metamorphosis in Xenopus? Does an induced memory during larval stages persist through metamorphosis?

3) Does the administration of the immunosuppressive drug cyclophosphamide (CY) facilitate the induction of tolerance? Does CY affect primary and/or memory responses?

4) When tolerance is induced in Xenopus, is it adoptively transferrable (i.e. is it passed on by transfer of viable cells from tolerant animals to non-tolerant ones?).

- C H A P T E R T W O -

- C H A P T E R T W O -

GENERAL MATERIALS AND METHODS

2.1. Animals and animal care:-

The animals used for the experiments of this study (Xenopus laevis) were bred and reared in the laboratory although their parents may have come from outside sources. Mating was induced by injecting adult females with 500 units and adult males with 100 units of chorionic gonadotrophin (Chorulon, Intervet) into their dorsal lymph sac. The pair were then placed in a tank of shallow, clean standing water with clean pieces of nylon gauze and left in a dark and quiet place over night. Next morning the gauze pieces with eggs attached to them were collected and placed in a tank of fresh, standing water. The jerking embryos (larvae) were collected the following day, using a wide-mouth glass pipette and transferred to a tank of clean, fresh standing water. At this stage, approximately 100 larvae were placed together in 8-10 litres of water in one tank. As they grew larger their numbers were reduced to 20-30 tadpoles per tank. From four days post-fertilization and until the metamorphosis climax, tadpoles were fed with nettle powder. During the metamorphosis climax, the animals usually stop feeding, whereas after the end of this period the new young toadlets become carnivorous and were fed on tubifex worms. Larger toadlets and older toads were kept 4 per tank of 6-8 litres, while toads were kept 2 per tank. All animals were kept at room temperature ($20^0 \pm 2^0$C) and were cleaned at least once a week.

Developmental staging of tadpoles was determined by their external morphological criteria according to the "Normal table of Xenopus laevis (Daudin)" (Nieuwkoop and Faber, 1967), (Figure 1).

The animals came from two different colonies in terms of histocompatibility. The members of one colony were histocompatible since they would accept tissue grafts from each other (Katagiri, 1978) and are referred to as G Xenopus. The mainspring parents of this colony were kindly supplied by Dr. Katagiri (Hokkaido University, Japan). The other colony of animals was of histoincompatible individuals which would reject each other's grafts.

G Xenopus animals were used when possible histocompatibility reactions between the cells of two individuals needed to be minimized. Such experiments are reported in Chapters 3, 4 and 6. These occasions were when pooling of spleen cells from several tadpoles was required for immunocytoadherence assays (Chapters 3 and 4). Also when adoptive transfer of induced immunological memory was attempted by the innoculation of spleen and thymus cells (Chapter 6).

2.2. Phosphate buffered saline:-

Two aqueous solutions were initially prepared of 0.15 M Potassium di-hydrogen phosphate (Solution A) (20.530 $g.dm^{-3}$ of KH_2PO_4, BDH) and 0.15 M Di-Sodium hydrogen phosphate (Solution B) (21.2550 $g.dm^{-3}$ of Na_2HPO_4 or 53.7 $g.dm^{-3}$ of $Na_2HPO_4.12H_2O$, BDH). These solutions were blended in certain proportions with saline depending on the pH of the buffer required. The buffers used in the experiments reported in this thesis were either of pH 7.2 or of pH 6.4. For pH 7.2 buffered saline

24 cm^3 of solution (A) and 76 cm^3 of solution (B) were added to each 100 cm^3 of saline. For pH 6.4 buffered saline 67.8 cm^3 of solution (A) and 32.2 cm^3 of solution (B) were added to every 100 cm^3 of saline. The pH was always checked using an electronic pH meter, and the necessary adjustments were made by adding more of the appropriate solution. Solution (A) is acidic and decreases the pH whereas solution (B) is alkaline and increases the pH. These solutions were kept at $4^{o}C$ until required.

2.3. Culture medium:

All solutions used in the preparation of culture media were kept on ice during the preparative stages of the work.

The medium used was the mammalian cell culture medium Leibovitz-15 (L-15) (Gibco, Bioculture). For preparation of cell suspensions 1 volume of distilled water was added to every 2 volumes of L-15 medium. For purposes of longer cell culture the L-15 medium was adjusted following the receipe of Manning and Botham (1980) for Xenopus cells. The final medium consisted of L-15 diluted with double distilled water, 0.01 M Hepes buffer (Gibco), 0.01 M Sodium biocarbonate 0.00005 M 2-Mercaptoethanol, 1% heat inactivated foetal calf serum (Gibco), 100 units.cm^{-3} pencillin, 100mg.cm^{-3} streptomycim and supplemented with 0.5% pooled and heat inactivated Xenopus serum.

2.4. Routes of injection:

2.4(i) In larvae:

Larvae were selected at the required stages of their development according to their external criteria (Nieuwkoop and Faber, 1967).

They were anaesthetized in 1:5000 (weight to volume) MS222 solution (Sandoz; or tricaine methane sulphonate, Sigma) and placed on a wet thin piece of foam under a dissecting microscope. The animals were constantly kept wet to avoid death by dehydration.

1) Intraperitoneal injection:-

The material to be injected was delivered by a micrometer syringe (Agla) through a number 30 gauge, sterile needle inserted from the dorsal side of the animal through the thin skin and the thick muscle at the base of the tail into the abdominal cavity. Three to 10 μm^3 of the required dose of material was injected in each tadpole, depending on the weight of the animal or requirements of the experiment. When allogeneic cells were injected, a stainless steel needle was used whereas when human gamma globulin solution, cyclophosphamide solution or sheep erythrocyte suspension were injected, ultra fine glass needles were prepared and used as described below.

2) Intramuscular injection:-

Intramuscular injections were given into the epaxial somatic musculature of the tail.

3) Intrathymic injection:-

The needle was inserted through the thin transparent skin overlying the thymic region and into the thymus where the injected material was delivered. The thymus was observed to swell due to the volume delivered then return to its original shape after delivery.

The ultra-fine glass needles used for these injections were prepared by thermally pulling a capillary tube, breaking the fine tip at a diameter between 20-50 μm. This tip was then sharpened on a fine air-driven sharpening stone and the needles were washed and siliconized to reduce the adherence of cells to their walls. These needles were connected to the micrometer syringe via a fine silicon tube, and fastened to a micromanipulator (Prior, right handed) by a tight-fitting plastic tube to permit the needle to be inserted with precision into the delicate tissues of the tadpoles.

2.4(ii) In toadlets and adult toads:-

Toadlets and adult toads were anaesthetized and placed on a bed of damp cotton wool.

1) Intraperitoneal injection:

The material was injected by inserting a hypodermic needle through the skin and abdominal muscles into the peritoneal cavity.

2) Via the dorsal lymph sac:

A long hypodermic needle was passed under the skin of the thigh of the toad to reach and penetrate the posterior septum of the dorsal lymph sac.

2.5 Statistics

For statistical analysis, Mann Whitney U test was used as described by Campbell (1974) (Campbell, R.C. 1974 "Statistics for Biologists" Second Edition. Cambridge University Press, London) and Witte (1980) (Witte, R.S. 1980 "Statistics". Holt, Rinehart and Winston Publishers, New York).

- C H A P T E R T H R E E -

- C H A P T E R T H R E E -

ONTOGENY OF ROSETTE FORMING CELL MEMORY RESPONSE TO SHEEP ERYTHROCYTES IN XENOPUS LAEVIS

3.1 INTRODUCTION

The immune system of anuran tadpoles has been shown to respond to a variety of antigens (Du Pasquier, 1967; Horton, 1969; Kidder et al., 1973; Moticka et al., 1973; Du Pasquier and Haimovich, 1974, 1976; Nagata, 1976). Some of these antigens are soluble such as human gamma globulin (HGG), bovine serum albumin (BSA) and keyhole limpet haemocyamin (KLH) and others are particulate such as foreign erythrocytes (foreign RBC), bacteria and hapten conjugated erythrocytes. Foreign RBC are examples of non-viable cellular antigens, the responses to which are probably the second most studied non-pathogenic particulate antigen in Xenopus laevis, the first being allogeneic tissues. Foreign RBC injected intraperitoneally were observed to undergo phagocytosis by peritoneal macrophages (Turner, 1970). The attainment of peak levels of rosette forming cells (RFC) and plaque forming cells (PFC) in the spleens of toads in response to an injection of foreign RBC was achieved between 7-12 days after immunization (Amirante, 1968; Du Pasquier, 1970; Kidder et al., 1973; Moticka et al., 1973; Horton et al., 1976; Wright and Cooper, 1980). Antibody production to foreign RBC however, was reported to occur later at two weeks post-immunization (Moticka et al., 1973; Turner, 1973; Horton et al., 1976; Nagata, 1976). Such antibodies were found to belong almost exclusively to the heavy molecular weight class of

immunoglobulins (IgM) with characteristics similar to the mammalian IgM (Moticka et al., 1973; Turner and Manning, 1974; Manning and Collie, 1975). Experiments on Xenopus laevis toads thymectomized at early larval stages revealed that their responses to foreign RBC are thymus-dependent (Horton et al., 1974, 1977; Turner and Manning, 1974; Manning and Collie, 1975, 1977; Tochinai and Katagiri, 1975). Further experiments on the restoration of thymectomized toads by implants of allogeneic and isogeneic thymus cells indicated the lack of histocompatibility restriction of Xenopus laevis responses to foreign RBC (Horton and Horton, 1975; Tochinai et al., 1976; Nagata and Tochinai, 1978; Katagiri et al., 1980; Kawahara et al., 1980; Nagata, 1980).

Unlike adults, anuran tadpoles are thought not to be able to respond anamnestically to foreign RBC. The induction of memory immune responses to foreign RBC in bullfrog tadpoles (Rana catesbeiana) was found to be unsuccessful unless the animals were subjected to an exhaustive course of immunization (Moticka et al., 1973). This led the authors (Moticka et al., 1973) to believe that these tadpoles are unable to mount a true anamnestic response to foreign erythrocytes. Post-metamorphic Xenopus laevis toadlets and Rana pipiens frogs on the other hand were reported to mount memory responses to foreign RBC (Kidder et al., 1973; Koppenheffer and Inchalik, 1979; Wright and Cooper, 1980). These secondary responses were characterised by earlier and higher levels of both RFC and serum antibodies.

One of the changes which occur during anuran metamorphosis is the decline in both structural and functional aspects of their immune system. For example, skin allograft rejection becomes less efficient,

if not abrogated (Cooper, 1973, 1976; Du Pasquier, 1973, 1976, 1982). It was also found that primary antibody responses to foreign RBC in tadpoles of Rana catesbeiana undergoing metamorphosis were much lower than those of younger tadpoles (Moticka et al., 1973). Moreover, larval spleen cells of Xenopus laevis were reported to respond to isogeneic adult cells in vitro and vice versa, disclosing some heterogeneity between the tissue antigens of tadpoles and adults. This is speculatively related to some changes in the histocompatibility system occurring during metamorphosis (Kobel and Du Pasquier, 1977; Du Pasquier, 1979). More recently, the metamorphic urodele Ambystoma mexicanum was reported to undergo similar histocompatible changes (Tahan and Jurd, 1983).

There have been reports that immunological memory to viable alloantigens (skin grafts) and soluble antigens (dinitrophenyl conjugated KLH) persists through metamorphosis in Xenopus laevis (Du Pasquier and Haimovich, 1976; Nagata, 1976; DiMarzo and Cohen, 1979; Manning and Botham, 1979; Botham and Manning, 1980; Cohen et al., 1980a, 1980b). However, it is not certain, particularly in the case of viable grafts, whether it is only the antigen which persists through metamorhposis. Foreign RBC in this respect represent a non-living particulate antigen which is not expected to thrive or persist in the circulation and tissues of the host.

Tolerance to foreign RBC was reported to be inducible in chicken (Mitchison, 1962) and mice (McCullaugh, 1973, 1974). In both cases the antigen was given either during embryonic stages (chicken) or at birth (neonatal mice). This is in agreement with the belief that immature and

developing embryos and neonates are more susceptible to tolerance induction than mature and developed adults (Burnet, 1959, 1962; Dresser and Mitchison, 1968; Solomon, 1971; Stocker and Nossal, 1977; Elson et al., 1979). The experiments detailed in this chapter form an ontogenetic investigation of immunological memory to sheep erythrocytes (SRBC) in Xenopus laevis in an attempt to answer the following questions:-

1) Are tadpoles capable of anamnestically responding to SRBC?
2) How does metamorphosis affect immunological memory induced during the larval period?
3) Is it possible to induce tolerance to SRBC by early larval exposure to the antigen?

3.2 MATERIALS AND METHODS

3.2.1 Preparation of sheep erythrocytes:-

Sheep erythrocytes (SRBC) obtained in Alsever's solution (Gibco) were washed at least three times in 0.85% saline before use. The supernatant at each time was replaced by fresh saline and the resuspended cells were spun for 5 minutes at 2200g. Following the third wash, the pellet containing approximately 2.5×10^{10} erythrocytes.cm^3 (100% SRBC suspension) was resuspended in saline to the required concentration.

3.2.2 Immunization of animals:-

All injections were administered intraperitoneally each containing a dose of 3.11×10^8 erythrocytes for each gram of body weight. Toads and older toadlets received the SRBC dose in 50 $\mu l.g^{-1}$ body weight from

a 25% suspension, whereas for tadpoles and young toadlets (less than one month old) the suspension was adjusted to 33.3% and 37.5% $\mu l.g^{-1}$ body weight were injected. All animals were slightly anaesthetised before receiving the injections.

3.2.3 Immunocytoadherence assay (ICA):-

The technique is similar to that used by Ruben and his group (1974). Cell suspensions were prepared on ice from spleens taken from heavily anaesthetised animals. The suspensions were prepared in diluted L-15 medium (Leibovitz-15, Gibco) supplemented with foetal calf serum (5 parts L-15: 4 parts double distilled water: 1 part heat inactivated foetal calf serum (Gibco)). A single spleen was used to provide enough cells for each test except when the spleens were small, then the spleens of 2-7 animals were pooled. This was the case when tadpoles or very young toadlets were used and in order to minimize reactions between the cells of different spleens, histocompatible G Xenopus were used.

The suspensions were washed at least once then resuspended to a concentration of 4-7 x 10^6 leucocytes.cm^{-3}. Two Eppendorf tubes were set for each test suspension. In each a $100cm^{-3}$ of spleen cell suspension were mixed with 20 μl of a 1% SRBC suspension. The tubes were then incubated over night (approximately 15 hours) in the refrigerator ($4^{o}C$). On the following morning each tube was mixed with 5 μl of 1% Nigrosin solution (for preparation see: Mishell and Shiigi, 1980) to determine the viability of spleen leucocytes. The suspensions were agitated by very gentle shaking or rotation on the surface of fine ice. The rosettes, viable and non-viable leucocytes, were counted under

a light microscope using a haemocytometer. Both chambers of the haemocytometer were filled from one tube and a leucocyte with at least three erythrocytes adhering to it's surface was considered as a rosette forming cell (RFC). The results are expressed as numbers of RFC.10^{-6} viable spleen leucocytes.

3.2.4 Serum preparation:-

Serum was prepared only from adult toads since tadpoles were too small to provide enough serum for the test (haemagglutination assay). The heart of the anaesthetized animal was exposed and the tip of it's ventricle was chipped by a sharp pair of scissors. The flowing blood was quickly collected by a pasteur pipette and transferred to an Eppendorf tube. The tube was left at room temperature for one hour before it was transferred to the refrigerator (4^{o}C) allowing the clot to contract over night. Next morning, the tube was spun at a high speed and the supernatant (serum) was taken into a clean tube which was then incubated at 56^{o} C for 30 minutes (this procedure inactivates the heat-labile complement components of the serum). The serum was then allowed to cool before it was used in the test.

3.2.5 Haemagglutination assay (HA):-

This assay was performed to detect SRBC agglutinating antibodies in the test serum following the technique of Turner (1970). An aliquot of 50 µl of 0.85% saline was placed into each of the required wells of a microtitre plate (12 wells were used for each test serum). 50µl of the test serum were mixed with the contents of the first well from which serial dilutions were carried on throughout the next 11 wells. Fifty

microliters of 0.25% SRBC suspension were added to each well after which the plate was covered with scotch tape and vigorously rotated on a horizontal smooth surface. The plates were incubated at room temperature for 3 hours first then at 4°C for an additional 12 hour period. Following incubation the plates were read.

3.2.6 Experimental design:-

The protocol for the following experiments is summarized and illustrated in Figure 2 . All animals were tested by the ICA described above, 8 days after receiving the challenge dose of SRBC.

Three groups of experimental animals, one of tadpoles at Stage 54 (Nieuwkoop and Faber, 1967), another of toadlets (less than a month old) and the third of toads (more than 6 months of age) received an identical challenge dose of SRBC (groups 1, 4 and 7 in Figure 2 respectively) in order to determine their primary responses to the antigen. At the time of the test the tadpoles reached Stage 56 of Nieuwkoop and Faber (1967).

Group 2 were tadpoles which have been primed with SRBC at Stage 48 (Nieuwkoop and Faber, 1967) then challenged thirty days later with the same antigen at which time they were at Stage 54 (Nieuwkoop and Faber, 1967).

Group 5 were toadlets that had received two SRBC injections as tadpoles, one at Stage 48 (Nieuwkoop and Faber, 1967) and the other at Stage 54 (Nieuwkoop and Faber, 1967). Thirty days following the last injection the animals had metamorphosed and received a challenge dose

Figure 2 Experimental protocol for the study of the ontogeny of primary and memory (secondary) humoral responses in Xenopus laevis.

Key

S SRBC (sheep erythrocytes) injection (3.11×10^8 erythrocytes. g body weight^{-1}).

Ch Challenge SRBC injection (3.11×10^8 erythrocytes. g body weight^{-1}).

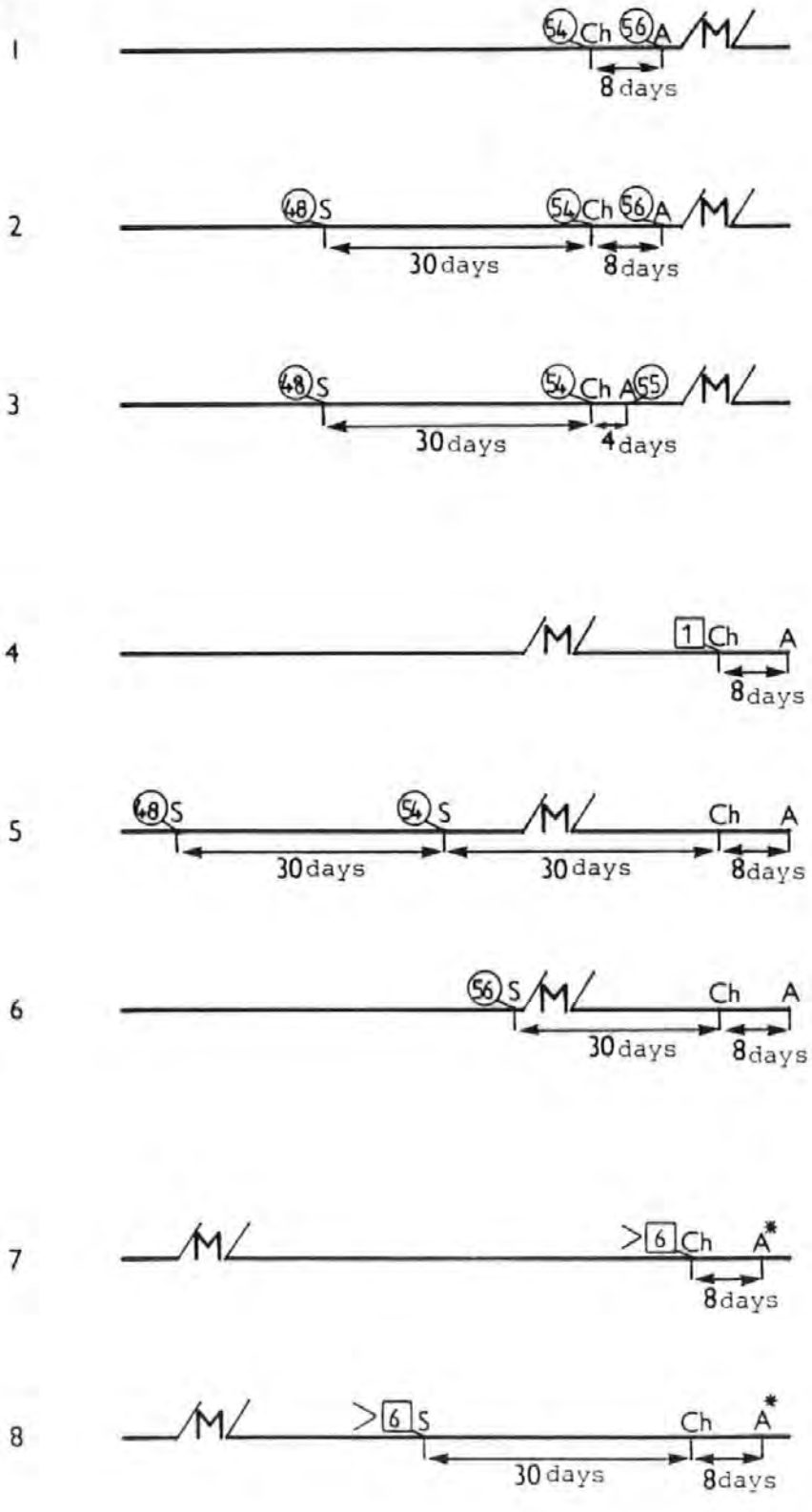
A ICA assay (immunocytoadherence assay). (*) Haemagglutination assay (HA) was performed in addition to ICA.

—/M/— Metamorphosis .

○ Stage of larval development (Nieuwkoop & Faber 1967); ⑱ , ⑭ , ⑮ , ⑯ larval stages.

□ Age in months post-metamorphosis. > more than.

Experiment No:



of SRBC.

Group 6 were inoculated with SRBC as tadpoles and challenged with the antigen thirty days later as toadlets.

The last group (8) were adult toads which were primed with SRBC and challenged thirty days later.

3.3. RESULTS:

3.3.1 Ontogeny of the primary RFC responses of Xenopus laevis to SRBC:

The data concerned with the ontogeny of the primary RFC production to SRBC in Xenopus laevis are given in Tables 1, 2 and 3 and are illustrated in Figure 3. Rosette forming cell levels in the spleens of adult toads were three times higher than those in the spleens of juvenile toadlets (38473 and 12500 mean splenic RFC.10^{-6} viable leucocytes respectively). Those of tadpoles were approximately equivalent to one third of those found in toadlets (4156 mean splenic RFC.10^{-6} viable leucocytes).

3.3.2 Ontogeny of RFC memory to SRBC:

A. RFC memory in tadpoles:

The data concerning RFC memory responses of tadpoles to SRBC are given in Table 1 and illustrated in Figure 3. The mean RFC production in tadpoles after receiving two doses of SRBC before they were tested was not significantly different from that which was produced after one

Group	Larval developmental stage at which SRBC was injected: Priming injection	Challenge	Day of test after challenge	RFC Numbers, 10^{-6} viable leucocytes: Individual readings	Mean ± S.E.
1	none	54*	8	3704,4274,4502	4156 ± 237
2	48*	54*	4	263,444,2220	976 ± 625
3	48*	54*	8	839,1268,3247,11565	4292 ± 2488

Table 1 The primary and secondary splenic rosette forming cell (RFC) responses in Xenopus laevis tadpoles to sheep erythrocytes (SRBC) (3.11×10^{8} cells.g body weight^{-1}).

*(48 and 54) larval stages (Nieuwkoop and Faber, 1967).

Each reading represents the result from a pool of spleens from 7-8 tadpoles. These results are from experiments 1, 2 and 3 in Figure 2.

Statistics (U Test)

Group 3~group 1 not significantly different

Group	Developmental Stage at which SRBC was injected: Priming injection	Challenge	RFC $.10^{-6}$ viable leucocytes: Individual readings	Mean $\pm$ S.E.
1	none	toadlet	1728,7524,12794, 19425,21026	12500 $\pm$ 3617
2	tadpole (48 & 54)	toadlet	1905*,2523*,4408, 4953*,5556*,9500, 21477,25350*,27671, 30952,34328*,40319*, 58868,61289,62074, 65065,89552	32120 $\pm$ 6536
3	tadpole (56)	toadlet	41853,48308,49469, 61247,67157,74242, 92859	62200 $\pm$ 6199

Table 2 The primary and secondary splenic rosette forming cell (RFC) responses in toadlets of Xenopus laevis to sheep erythrocytes (SRBC) (3.11×10^8 cells. g body weight^{-1}). The toadlets were less than a month old (post-metamorphosis). The tests were carried out 8 days after challenge. * Two spleens were pooled. These results are from experiments 4, 5 and 6 in Figure 2.

Statistics (U Test) Group 2 $\sim$ group 1 not significantly different
Group 3 $\sim$ group 2 not significantly different
Group 3 $>$ group 1 ρ 0.01

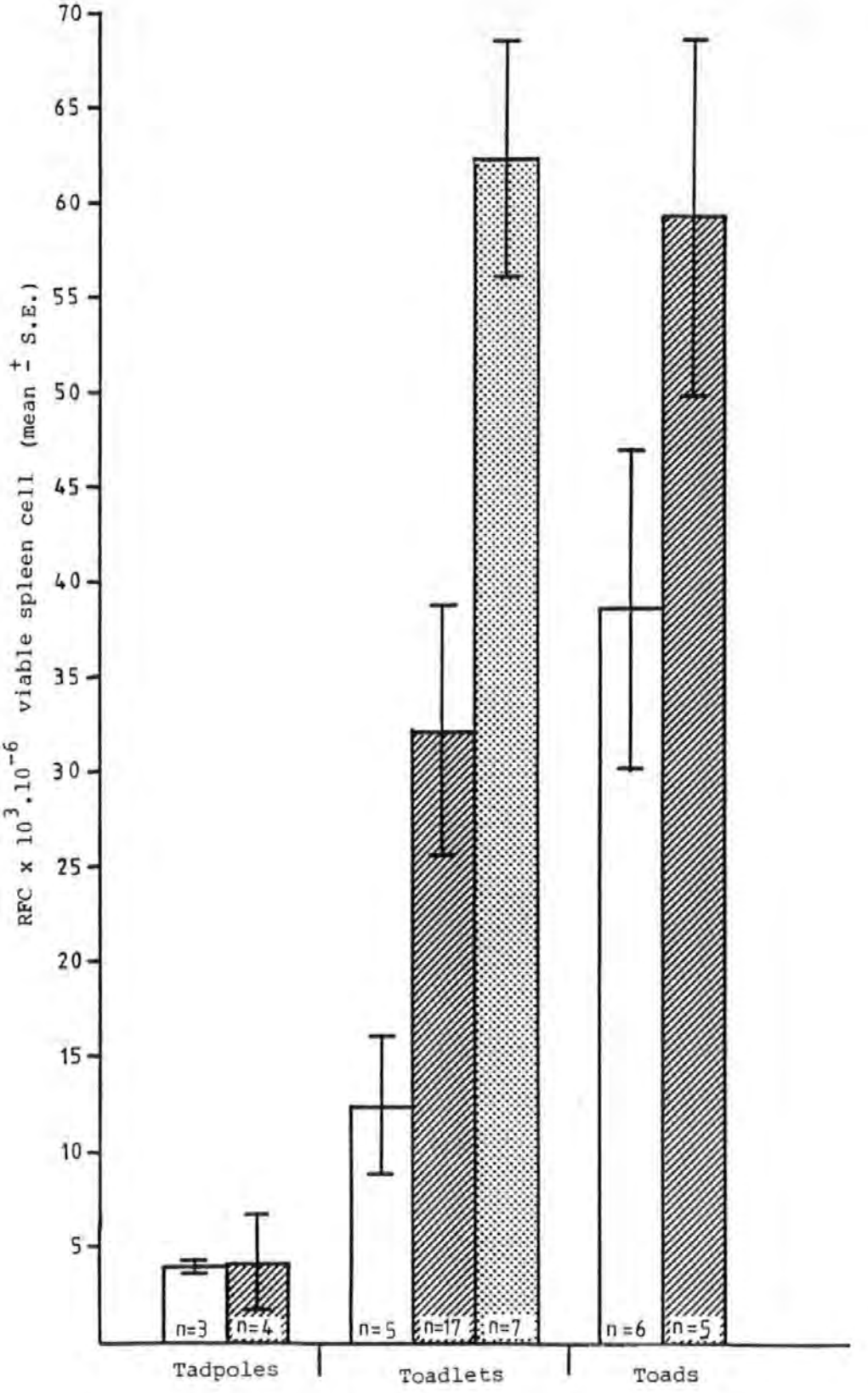
Group	SRBC injections given		RFC $.10^{-6}$ viable leucocytes		HA titres ($-\log_2$)	
	Priming	Challenge	Individual readings	Mean $\pm$ S.E.	Individual readings	Mean $\pm$ S.E.
1	None	Yes	2667		<1	
			29421		<1	
			38889		1	
			46281		2	
			56438		not done	
			57143	38473 $\pm$ 8354	not done	0.75 $\pm$ 0.48
2	Yes	Yes	35447		4	
			51300		5	
			52083		6	
			65993		7	
			90856	59136 $\pm$ 9288	7	5.8 $\pm$ 0.58

Table 3 The primary and secondary splenic rosette forming cell (RFC) and serum haemagglutinating antibody (HA) responses in adult Xenopus laevis toads to sheep erythrocytes (SRBC) (3.11×10^8 cells. g body weight^{-1}). The animals were tested for both responses on day 8 after challenge. These results are from Experiments 7 and 8 in Figure 2.

Statistics (U Test)

For RFC Group2 $\sim$ group 1 not significantly different. For HA group 2 $>$ group 1 $\rho 0.01$

Figure 3 The ontogeny of primary and secondary splenic rosette forming cell (RFC) responses in Xenopus laevis to sheep erythrocytes (SRBC) (3.11×10^{8} cell. g body weight^{-1}). In tadpoles for primary responses the animals were immunized at Stage 54 and tested by the immunocytoadherence assay (ICA) eight days later. For toadlets and toads, the animals were immunized then tested in ICA eight days later. For the secondary responses tadpoles were primed at Stage 48 and challenged 30 days later at Stage 54. Toadlets were primed at either larval Stages 48 and 56 or only at Stage 56, then challenged 30 days later as toadlets. Toads were primed, then challenged 30 days later. All animals were tested by ICA eight days after challenge. (n) number of animals in each group. These results are from experiments 1-8 in Figure 2.


70
65
60
55
50
45
40
35
30
25
20
15
10
5
RFC x $10^3.10^{-6}$ viable spleen cell (mean ± S.E.)
n=3
n=4
n=5
n=17
n=7
n=6
n=5
Tadpoles
Toadlets
Toads
Primary response
Secondary response
Secondary response in toadlets primed at Stage 56 only

injection (primary response) (Figure 3).

B. RFC memory in toadlets first immunized at an early larval stage:-

The results of these experiments are given in Table 2 and illustrated in Figure 3 . Toadlets which had been first immunized with SRBC at larval Stage 48 (Nieuwkoop and Faber, 1967) were found to produce a mean of 32120 splenic RFC.10^{-6} viable leucocytes after challenge. This was 2.6 times the primary RFC levels Figure 3.

C. RFC memory in toadlets immunized at a late larval stage:-

The results of these experiments are given in Table 2 and are illustrated in Figure 3 . Toadlets which had been first immunized with SRBC at larval Stage 56 (Nieuwkoop and Faber, 1967) produced a mean of 62200 splenic RFC.10^{-6} viable leucocytes after challenge. This was twice as high as those RFC levels produced in toadlets first immunized at larval Stage 48 and five times the primary RFC levels.

D. RFC memory in toads:-

The data concerning the RFC memory response of adult toads to SRBC are given in Table 3 and are illustrated in Figure 3 . The mean splenic RFC levels were 59136 RFC.10^{-6} viable leucocytes. This increase in RFC production over the primary levels in toads was lower than the increase in their agglutinating antibodies following secondary immunization, Figure 3. In fact, the secondary serum antibody levels were 7.7 times higher than those produced in the primary response.

3.4 DISCUSSION:

The results of the experiments detailed in this chapter appear to suggest that anamnestic rosette forming cell (RFC) responses in Xenopus laevis to sheep erythrocytes (SRBC) cannot be evoked during the larval period of their development. These findings are consonant with those

reported for bullfrog tadpoles (Rana catesbeiana) in response to the same antigen (Moticka et al., 1973). This may be due to an inefficient and immature memory mechanism. Further experiments described in this chapter, however, have shown that memory to immunization with SRBC has been induced when the animals were tested after metamorphosis as toadlets. Unprimed toadlets show lower primary RFC levels. This is also supported by recent reports that memory to the hapten, dinitrophenyl conjugated to keyhole limpet hemocyanin (DNP-KLH) (Du Pasquier and Haimovich, 1976) and to alloantigens (skin allografts) (Nagata, 1976) could be initiated in Xenopus laevis tadpoles but expressed after metamorphosis; the memory responses were not tested before metamorphosis (reviewed by Du Pasquier, 1982). Another possible factor which may result in obscuring the occurence of a memory response is a long period between the first and the second antigen injections. The time interval between the first and the second SRBC immunization (30 days) which was used in the present study was sufficient for memory to develop in toadlets and toads. Moreover, this time interval also corresponds to that used by other workers to induce anamnestic secondary responses to SRBC in Xenopus laevis toadlets (Kidder et al., 1973; Koppenheffer and Inchalik, 1979). It therefore appears that there may be another factor that is affecting the expression of immunological memory in Xenopus laevis tadpoles. This is thought to be related to the lower efficiency of tadpoles in the production of low molecular weight antibodies (LMW).

Upon secondary immunization, adult toads show an increase in their anti-SRBC agglutinating antibody levels. Their RFC levels, however, were not significantly increased, instead primary levels were attained. These observations were unexpected as other studies have shown that anamnestic

secondary reactions in adult anurans are generally characterized by higher and/or earlier responses (Simnet, 1965; Lin et al., 1971; Kidder et al., 1973; Du Pasquier, 1975; Azzolina, 1976; Du Pasquier and Haimovich, 1976; Koppenheffer and Inchalik, 1979; Wright and Cooper, 1980). Nevertheless, Minagawa et al. (1975) have also recorded similar observations to these reported here. They have found that adult Rana catesbeiana failed to elicit augmented plaque forming cell (PFC) levels in response to a second immunization with SRBC although they noticed an abundance of plasma cells in various lymphoid organs (possibly indicating active antibody production). In another report, adults of Bufo marinus have also failed to produce increased secondary antibody levels in response to high doses of polymerized flagellin (Azzolina, 1976).

Bullfrog larvae which were unable to elicit a "true" anamnestic secondary response to SRBC, produced almost exclusively high molecular weight immunoglobulins (IgM antibody) in response to the second injection of SRBC (Moticka et al., 1973). Xenopus laevis tadpoles (Stages 53-56) were unable to produce good levels of low molecular weight immunoglobulin (LMW) following a secondary immunization with either human immunoglobulin light chain or Limulus hemocyanin (Jurd et al., 1975). Thus it seems that the inability of tadpoles to produce anamnestic memory RFC response may be related to their poor LMW production which is also of low affinity (Du Pasquier, 1973; Du Pasquier and Haimovich, 1974, 1976; also reviewed by Du Pasquier, 1982); both functions may be under common control.

Adult anurans are known to have an efficient system of antibody production (Marchalonis and Edelman, 1966; Hadji-Azimi, 1971; Marchalonis, 1971; Du Pasquier and Haimovich, 1974, 1976; Jurd et al., 1975; Azzolina,

1976; Pross and Rowlands, 1976; Marchalonis and Germain, 1980; also reviewed by Du Pasquier, 1973, 1976, 1982; Cooper, 1976; Hadji-Azimi, 1979). The production of antibodies may be considered as a stronger and more efficient mechanism for the elimination or apprehension of antigen than the RFC response in Xenopus laevis toads. In mice recently, some evidence was found that B cells forming rosettes did not synthesise immunoglobulin in response to poke weed mitogen in vitro (Davey et al., 1983). This also implies that there may not be a real difference between the so called "secretory" and "non secretory" antigen binding cells or RFC in Xenopus laevis and Rana pipiens (Rimmer, 1976; Edwards et al., 1976; see also Edwards and Ruben, 1982). "Non secretory" RFC were found to bind one layer of RBC whereas "secretory" cells were found to bind two layers or more. The observations of these authors may be due to pseudopod-like extensions of the RFC and may occur to all RFC at a certain stage of their formation and at certain culture conditions such as the temperature. In mammals the "secretory" RFC required 37^{o}C while the "non secretory" ones were very fragile and were best demonstrated at 4^{o}C (Haskill et al., 1972).

In the present study, although the levels of splenic RFC in the primary response of Xenopus laevis to SRBC seems to increase proportionally with age, in the secondary response they seem to be significantly elevated (over primary levels) only in toadlets. These toadlets were primed as tadpoles and it may be considered that metamorphosis had an enhancing effect on the responses. This however, seems to be doubtful since Xenopus laevis toadlets can elicit an anamnestic secondary response to SRBC (Kidder et al., 1973; Koppenheffer and Inchalik, 1979), while as shown in the above experiments both tadpoles and adults failed to produce higher secondary RFC levels.

As mentioned above, memory to SRBC induced in tadpoles was expressed after metamorphosis. This seems to indicate that immunological memory induced in tadpoles persists through the morphological, physiological, biochemical and behavioural changes which occur during metamorphosis. These conclusions are in agreement with those made for Rana catesbeiana and Xenopus laevis for their antibody response to DNP-KLH (Du Pasquier, 1975; Du Pasquier and Haimovich, 1976) and in Xenopus laevis for their allograft rejection (Nagata, 1976).

Arguably, it could be suggested that during the anuran larval period, a process of suppression affects the expression of immunological memory. This process is then counter-affected by a non-specific suppressive factor(s) during metamorphosis. Being non-specific this factor(s), which may be a hormonal or cellular one(s), has the ability to also suppress and deplete other cell clones. According to this theory, the unsensitized all-reactive clones would be expected to suffer great losses in their numbers and thus become sensitive to tolerance induction. On the other hand, those sensitized and expanded clones would be expected to suffer relatively smaller losses and survive through metamorphosis. Cell depletion does occur, particularly in the thymus and spleen (Du Pasquier and Weiss, 1973; Du Pasquier, 1976, 1982). Furthermore, DNP-KLH primed tadpoles express larval antibody electro-focusing bands upon challenge with the same antigen after metamorphosis, while animals primed and challenged as toadlets could not reproduce these larval bands (Du Pasquier et al., 1979). At metamorphosis, unsensitized Xenopus laevis individuals may be rendered tolerant to allografts (Chardonnens and Du Pasquier, 1973; Cohen et al., 1980; Barlow et al., 1981). In contrast, animals exposed to alloantigens as larvae (having rejected an allograft) resisted

tolerance induction to the same allotype (Du Pasquier, 1982). In another report similar animals which had rejected an allograft as tadpoles, rejected a second graft in a second set fashion (Nagata, 1976) when applied after metamorphosis. This alone however, does not provide a satisfactory explanation to the emergence of memory expression only after metamorphosis and not during the larval period. An additional factor(s) thus seem to be involved. The experiments detailed in the present study seem to suggest the development of such a factor(s) during metamorphosis of Xenopus laevis which may link between the persisting memory "cells" and effector RFC clones. Furthermore, this factor(s) seems to be ineffective when the animal receives the primary SRBC injection at an early larval stage (Stage 48). Thus leading to the assumption that precursors of this factor(s) have been modified at this early stage when most thymus dependent responses seem to be vulnerable to thymectomy. The "factor" could, of course, have a morphological basis. For example, it is known that the sites concerned with antigen trapping (the splenic dendritic cells) do not attain their mature location and morphology until about Stage 59 (Baldwin and Sminia, 1982).

- C H A P T E R F O U R -

- C H A P T E R F O U R -

THE INFLUENCE OF CYCLOPHOSPHAMIDE ON THE ONTOGENY OF THE MEMORY RESPONSE IN XENOPUS LAEVIS TO SHEEP ERYTHROCYTES.

4.1 INTRODUCTION

Experiments in the previous chapter (Chapter 3) appear to indicate that the immune system of larval Xenopus laevis was already too mature to facilitate the induction of immunological tolerance to sheep erythrocytes (SRBC). In this chapter, an attempt will be made to promote the induction of tolerance to SRBC by chemically suppressing the immunologically competent system of larval and adult Xenopus laevis.

In higher vertebrates it is easier to induce tolerance to an antigen in animals having immature or partly incapacitated immune systems (Solomon, 1971; Stocker and Nossal, 1977). Examples of this are tolerance induced in foetal and neonatal animals (McCullagh, 1974; Hunneyball and Stranworth, 1979; Etlinger and Chiller, 1979) and also that induced in irradiated animals (Gershon and Kondo, 1971; Bains and Sundaram, 1979). Chemical incapacitation of the immune response is also possible by the use of immunosuppressive agents, such as cyclophosphamide (CY) (Aisenberg and Davis, 1968; Dietrich and Dukor, 1969; Many and Schwarts, 1970).

Cyclophosphamide is a chemically synthesised drug and is one of the most potent and widely used alkylating agents in chemotherapy. It is known to disrupt DNA synthesis during cell division as well as other active cellular processes such as protein synthesis in non-dividing cells

(Foley et al., 1961; Bach, 1975). Animals treated with CY, therefore, suffer great losses in their cell numbers, especially those in actively dividing tissues (Turk and Poulter, 1972; Poulter and Turk, 1972). This depletion is not long lasting and recovery is attributed to cytoautorepair rather than to proliferation of precursor or stem cells (Berenbaum et al., 1973; Shand, 1979).

The treatment of animals with CY modified their responses to various antigens. Although in most cases these responses were suppressed (Berenbaum and Brown, 1964; Bach, 1975; Botzenhardt and Lemmel, 1975; Gagnon and Maclennan,1981), some were enhanced (Turk, 1973; Polak, 1977). These effects were also found to depend on the temporal relationship between immunization and CY administration (Gras and Tillo, 1981).

Although CY is inactive *in vitro per se* (Foley et al., 1961) it can be activated by pretreatment with liver enzymes, thus affecting *in vitro* responses in a similar way to its effects *in vivo* (Berenbaum et al., 1973; Connors et al., 1974). Cyclophosphamide has recently been shown to suppress the humoral immune response of an anuran amphibian (*Rana pipiens*) to the hapten trinitrophenyl (TNP) conjugated to the bacterial lipopolysaccharide (LPS) as a carrier (Bugbee et al., 1983). In this chapter, the effects of CY on the primary and secondary (anamnestic) response of *Xenopus laevis* during ontogeny will be investigated. This will include an attempt to induce tolerance to sheep erythrocytes (a nonviable particulate antigen) following treatment with this immunosuppressive drug.

4.2 MATERIALS AND METHODS

The preparation of suspensions of sheep erythrocytes (SRBC), immunization of the animals, immunocytoadherence assays (ICA), preparation of sera and haemagglutination assays were performed as described in the previous chapter (Chapter 3).

4.2.1 Preparation of cyclophosphamide (CY) solutions and dosage:

Immediately before use, CY (Sigma) was dissolved in 0.85% saline. When this drug was injected alone it was made up to $6mg.cm^{-3}$ for adults and toadlets and to $4mg.cm^{-3}$ for tadpoles. Adults and toadlets received $50\mu l.g^{-1}$ body weight whereas $37.5\mu l.g^{-1}$ body weight were given to tadpoles from the respective CY preparation.

When CY was injected with SRBC into toadlets and toads, one volume of $12mg.cm^{-3}$ CY was mixed with an equal volume of 50% SRBC suspension and given at $50\mu l.g^{-1}$ body weight. For tadpoles however, $8mg.cm^{-3}$ CY were mixed with an equal volume of 66.6% SRBC suspension and injected at $37.5\mu l.g^{-1}$ body weight. With this procedure toadlets and toads received a dose of $300mg.g^{-1}$ body weight of CY whereas tadpoles received half this concentration because the higher dose was lethal to tadpoles.

4.2.2 Experimental design:

The protocol for the following experiments is summarized and illustrated in Figure 4. All animals were injected intraperitoneally and the number of rosette forming cells (RFC) in their spleens (an indication of the immune response) was estimated by the ICA technique

Figure 4 Diagramatic representation of the experimental protocol for the study of the influence of cyclophosphamide (CY) on the primary and memory (secondary) humoral responses to sheep erythrocytes (SRBC) during the ontogeny of Xenopus laevis. Tadpoles were given a dose of 150µg.g body weight^{-1} and toads were given 300µg.g body weight^{-1}.

Key:

S SRBC (sheep erythrocytes) injection (3.11 x 10^8 erythrocytes. g body weight^{-1})

Ch Challenge SRBC injection (3.11 x 10^8 erythrocytes. g body weight^{-1})

A ICA assay (immunocytoadherence assay). (*) Haemagglutination assay (HA) was performed in addition to ICA.

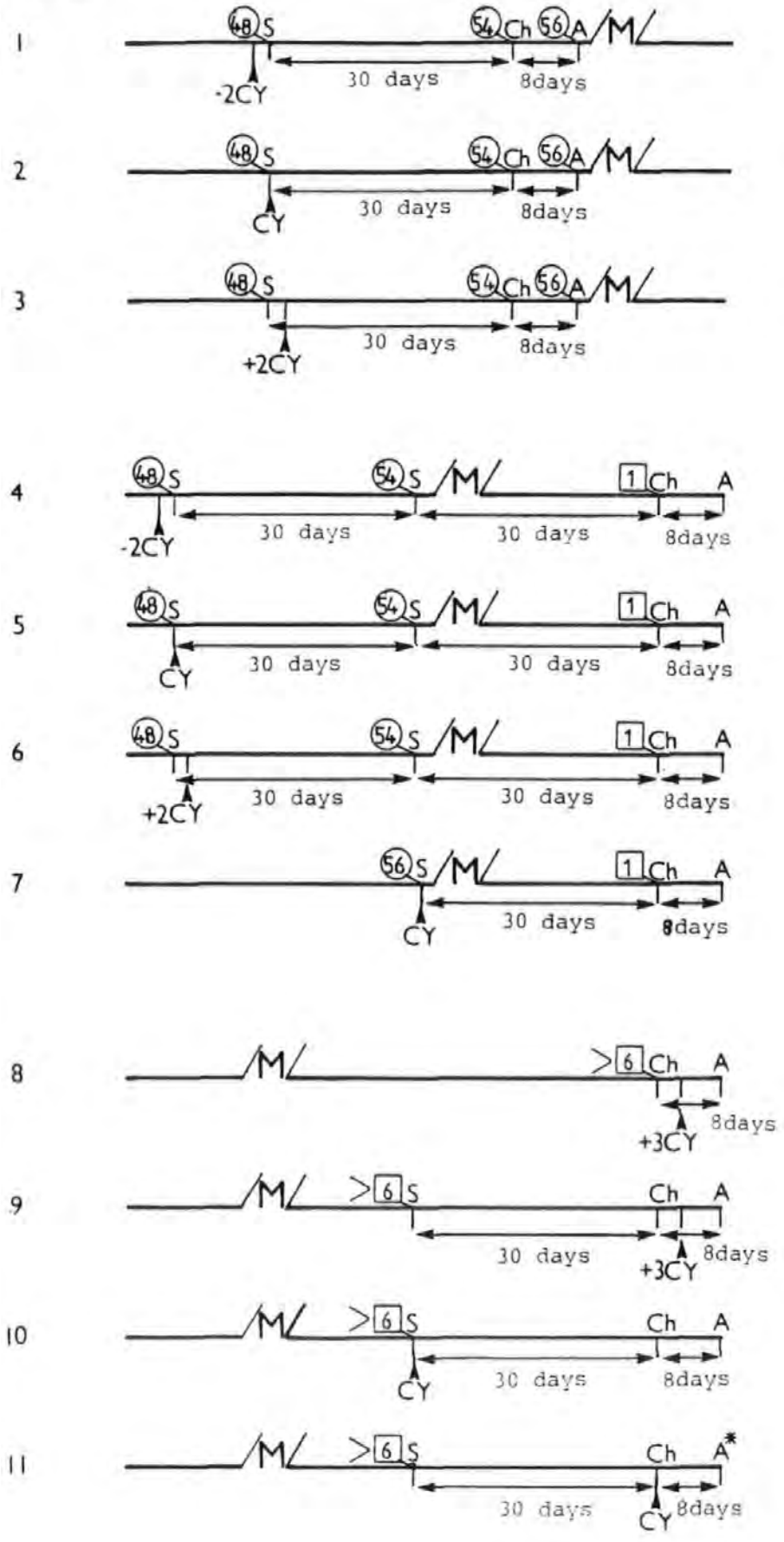
M Metamorphosis.

○ Stage of larval development (Nieuwkoop & Faber 1967). ㊽, ㊾, ㊿, (56) larval stages.

□ Age in months post-metamorphosis.> more than.

CY Stage when given either cyclophosphamide or a control (saline) injection. (-2,-3) two or three days earlier than the following SRBC injection (+2,+3) two or three days after the preceeding SRBC injection.

Experiment No:



8 days after receiving the challenge dose of SRBC.

Three groups of tadpoles at Stage 48 (Nieuwkoop and Faber, 1967) were injected with CY either two days before, on the same day or two days after receiving a dose of SRBC. Thirty days later the animals received a challenge dose of SRBC (Experiments 1, 2 and 3 in Figure 4 respectively). At the time of the test the tadpoles reached Stage 54. Another three groups of tadpoles were similarly treated at Stage 48, where CY was given two days before, on the same day or two days after receiving a dose of SRBC (Experiments 4, 5 and 6 in Figure 4 respectively). These animals were re-immunized thirty days later and thirty days after that they were given a challenge dose of SRBC. At the time of re-immunization the tadpoles reached Stage 54 and they had metamorphosed before receiving the challenge dose.

A final group of tadpoles received an injection of CY and SRBC at Stage 56 and, 30 days later, were given a challenge dose of SRBC. These animals had metamorphosed before they were given the challenge injection (Experiment 7 in Figure 4). Three groups of toads were given different doses of CY three days after receiving a challenge injection of SRBC. One group received a dose of $50\mu g.g^{-1}$ body weight of CY, the second group received $150\mu g.g^{-1}$ body weight and the third group group received $300\mu g.g^{-1}$ body weight of the drug (Experiment 8 in Figure 4). The body weights of these animals were recorded whenever the animals were injected and also when the animals were tested. A further three groups of toads were given an injection of SRBC on days 0 and, thirty days later, they were given the challenge dose. The three groups of animals had also received an injection of CY,

one group on day 0, the second on day 30 and the third on day 33 (Experiments 9, 10 and 11 in Figure 4 respectively). Serum taken from toads used in Experiment 11 (in Figure 4) was screened for haemagglutinating antibodies by the passive haemagglutination assay.

4.3 RESULTS

4.3.1 The influence of cyclophosphamide (CY) on the lymphocytic content in spleens of Xenopus laevis toads immunized with sheep erythrocytes (SRBC).

The results of experiments on the influence of CY on the lymphocytic content in the spleens of Xenopus laevis toads immunized with SRBC are summarized in Table 4. Cyclophosphamide was administered three days after an injection of the antigen (SRBC) (five days before the test).

At all three doses, 50μg, 150μg or 300μg.g^{-1} body weight, CY appears not to have had any significant effect on the mean body weight of toads immunized with SRBC. The injection of either 150μg or 300μg.g^{-1} body weight of CY however, reduced the ratio of splenic leucocytes to body weight in immunized toads (groups 5 and 6 compared with group 3 in Table 4; group 5 $<$ group 3 $\rho < 0.05$, group 6 $<$ group 3 $\rho < 0.005$). The lower dose of CY (50μg.g^{-1} body weight) does not however, appear to have had any significant effect on the number of splenic leucocytes per gram body weight of toads immunized with SRBC.

Group Number	Injected material Day 0	Injected material Day 3	Body Weight (grams) Day 0	Body Weight (grams) Day 3	Body Weight (grams) Day 8	Splenic leucocyte concentration ($x10^6$ leucocytes. ml^{-1} of medium	The number of splenic leucocytes per gram body weight ($x10^3$)	n
1	none	none	not done	not done	10.75 ± 1.4	7.375 ± 2.6	6.488 ± 1.2	4
2	Saline	Saline	11.13 ± 1.4	12.00 ± 2.1	9.88 ± 1.7	5.770 ± 0.5	6.345 ± 1.2	4
3	SRBC	Saline	9.42 ± 0.5	9.67 ± 1.1	8.17 ± 0.7	6.162 ± 0.2	7.850 ± 0.8	6
4	SRBC	CY 50	13.00 ± 1.4	12.25 ± 1.7	11.17 ± 1.4	6.442 ± 1.1	5.920 ± 0.8	6
5	SRBC	CY 150	12.33 ± 0.7	12.00 ± 0.7	10.50 ± 0.5	5.508 ± 0.9	5.188 ± 0.7	6
6	SRBC	CY 300	15.17 ± 1.01	14.50 ± 0.7	13.75 ± 0.8	5.230 ± 0.4	3.830 ± 0.4	5

Table 4 The effect of different doses of Cyclophosphamide (CY) on the body weights and relative number of splenic leucocytes in adult Xenopus laevis after immunization with sheep erythrocytes (SRBC). The animals were weighed before receiving the injections and before extracting their spleens. When the animals were killed on day 8, a cell suspension was made from each spleen in 1.5ml of medium and the number of leucocytes was counted using a haemocytometer. (SRBC) sheep erythrocytes injected at a dose of 3.11×10^8.g body $weight^{-1}$ (CY) cyclophosphamide given at a dose of either 50 or 150 or 300µg. g body $weight^{-1}$. (n) number of animals in each group. These results are from Experiment 8 in Figure 4.

Statistics (U Test) Comparisons of the numbers of splenic leucocytes per gram body weight.

Group 2 ~ group 3 not significantly different
Group 1 ~ group 3 not significantly different
Group 6 < group 3 $\rho < 0.005$

Group 1 ~ group 2 not significantly different
Group 4 ~ group 3 not significantly different
Group 5 < group 3 $\rho < 0.05$

4.3.2 The suppressive effect of Cyclophosphamide (CY) on the primary and memory rosette forming cell (RFC) responses in Xenopus laevis toads to sheep erythrocytes (SRBC):-

The data showing the suppressive effect of CY on the primary RFC responses in Xenopus laevis toads are given in Table 5 and are summarized and illustrated in Figure 5 . The data showing the suppressive effect of CY on the memory RFC response in Xenopus laevis toads, when given three days after the challenge SRBC injection, are summarized and illustrated in Figure 6.

These results clearly demonstrate the potency of CY in suppressing the RFC responses of Xenopus laevis toads to SRBC when given three days after the challenge injection of the antigen. This effect on the primary responses appears to be more prominent when the higher dose of the drug ($300\mu g.g^{-1}$ body weight) was used. This dose of CY was enough to suppress approximately 90% of the RFC response (in comparison with the responses of toads immunized with SRBC, but not given CY, group 6 compared with group 3 in Table 5 and Figure 5 , group 6 $<$ group 3 ρ 0.01). The memory RFC response in Xenopus laevis toads to SRBC was also suppressed when $300\mu g.g^{-1}$ body weight of CY were injected three days after the challenge antigen inoculation (group 2 $<$ group 1, $\rho<0.05$ in Figure 6).

4.3.3 Ontogeny of rosette forming cell (RFC) memory response to sheep erythrocytes (SRBC) in Xenopus laevis under treatment with cyclophosphamide (CY):-

The drug (CY) in these experiments was given with the first injection of the antigen (SRBC) unless otherwise specified.

Table 5 The effect of injecting different doses of cyclophosphamide (CY) on the primary splenic rosette forming cell (RFC) response in adult Xenopus laevis to sheep erythrocytes (SRBC). (SRBC) sheep erythrocytes injected at a dose of 3.11×10^8 cells. g body weight^{-1}. (CY) cyclophosphamide injected at a dose of either 50 or 150 or 300 μg.g body weight^{-1}. These results are from experiment 8 in Figure 4.

Statistics (U Test):

Group 5 < group 3	ρ 0.05
Group 6 < group 3	ρ 0.01
Group 4 ~ group 3	not significant

Group Number	Injections given		Individual readings of splenic RFC numbers. 10^6 viable leucocytes^{-1}	mean ± S.E.
	on day 0	on day 3		
1	none	none	0,0,0,0.	0
2	Saline	Saline	347,412,0,0.	190±110
3	SRBC	Saline	2667	
			29421	
			38889	34873±8359
			46281	
			56438	
			57143	
4	SRBC	CY 50	14815	
			15205	
			18182	27252±5003
			33766	
			35598	
			45946	
5	SRBC	CY 150	1802	
			4289	
			8333	9425±2450
			11358	
			12251	
			18519	
6	SRBC	CY 300	488	
			2222	
			2273	3972±1667
			3065	
			3783	
			12000	

Figure 5 The effect of injecting different doses of cyclophosphamide (CY) on the primary splenic rosette forming cell (RFC) response in Xenopus laevis toads to sheep erythrocytes (SRBC) (3.11×10^8 cells.g body weight^{-1}). (n) number of animals in each group. These results are from Experiment 8 in Figure 4.

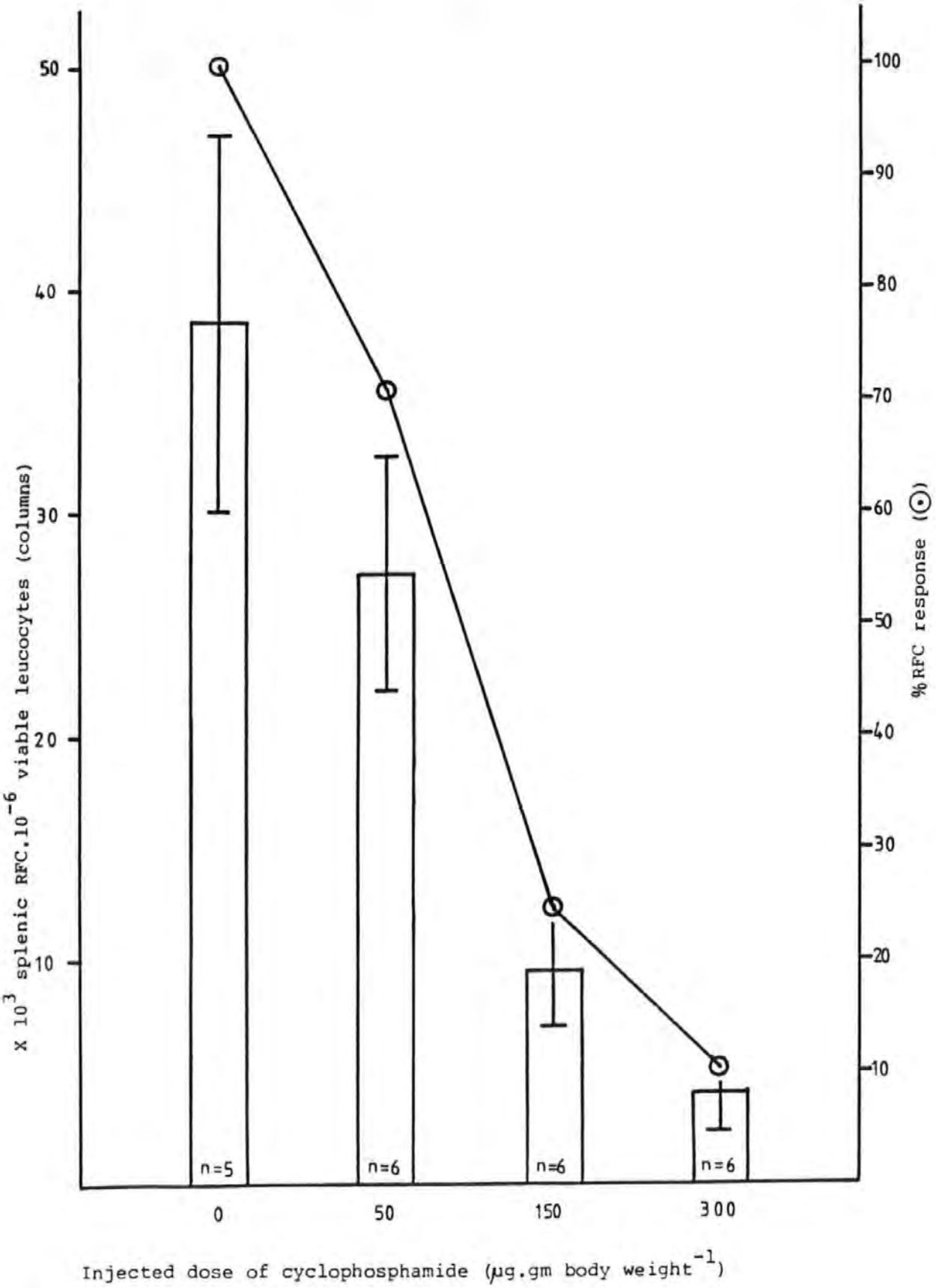
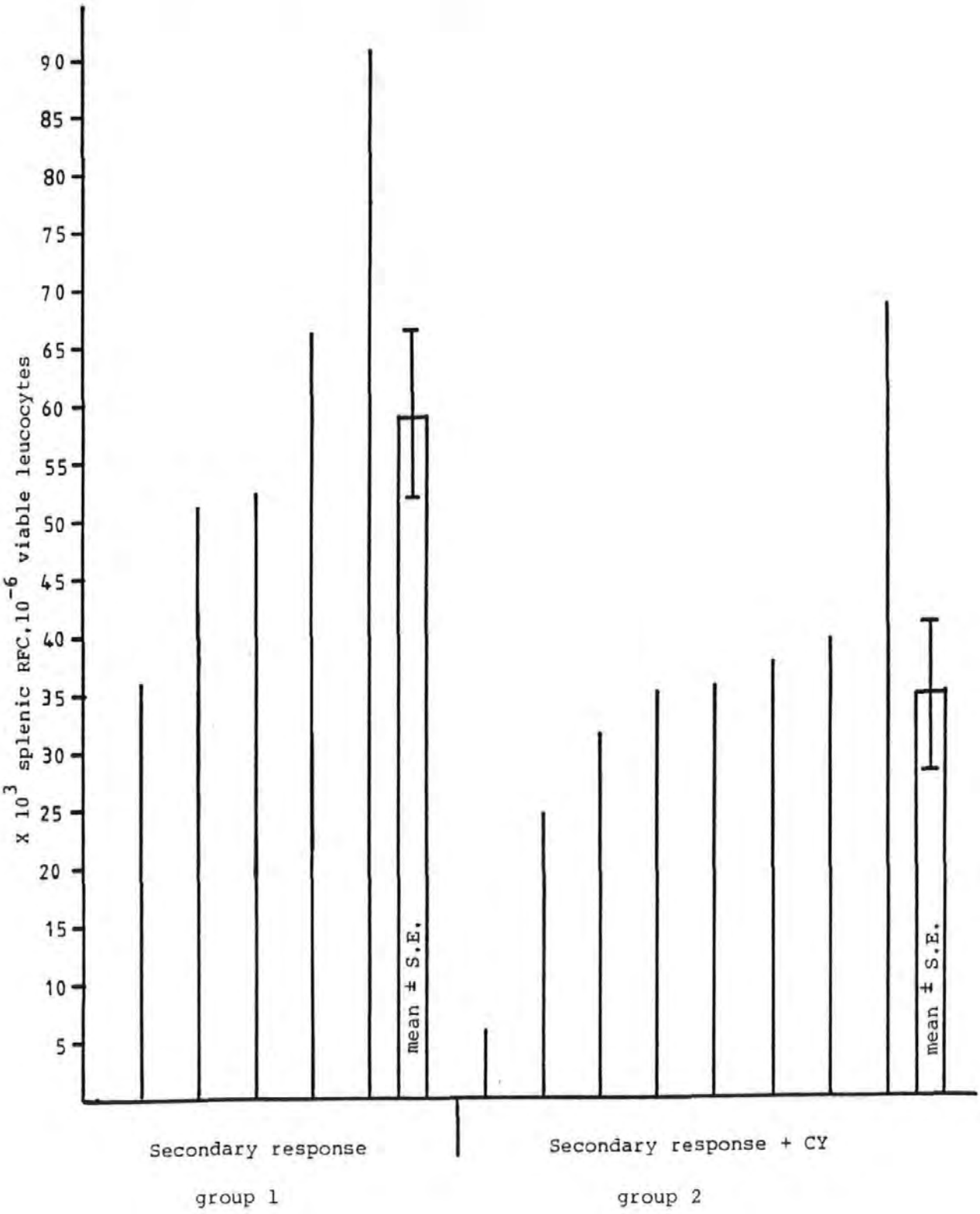

50
40
30
20
10
x 10^3 splenic RFC.10^{-6} viable leucocytes (columns)
100
90
80
70
60
50
40
30
20
10
% RFC response (⊙)
n=5
n=6
n=6
n=6
0
50
150
300
Injected dose of cyclophosphamide (μg.gm body weight^{-1})

Figure 6 The effect of cyclophosphamide (CY) on the secondary splenic rosette forming cell (RFC) response in Xenopus laevis toads to sheep erythrocytes (SRBC) (3.11×10^8 cells.g body weight^{-1}). A dose of 300µg CY. gm body weight^{-1} was injected three days after the second SRBC immunization. These results are from Experiment 9 in Figure 4. (Columns) mean $\pm$ S.E. Each line represents the result from one animal. The CY treatment suppressed approximately 41.2% of the response.


x 10^3 splenic RFC, 10^{-6} viable leucocytes
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
mean ± S.E.
mean ± S.E.
Secondary response
group 1
Secondary response + CY
group 2

A. RFC memory response in tadpoles:

The data concerning the RFC memory responses of tadpoles after treatment with CY are given in Table 6 . There is little difference in the mean RFC memory responses of tadpoles to SRBC irrespective of whether they received treatment with CY or not.

B. RFC memory response in toadlets:

(i) Toadlets first injected with SRBC at an early larval stage (Stage 48).

The results from these experiments are given in Table 7 and summarized and illustrated in Figure 7 . The administration of CY two days before the first SRBC injection does not appear to have significantly altered the mean RFC level in the memory response of toadlets to SRBC (in comparison with the memory response of toadlets not treated with CY; 29072 and 32120, mean splenic RFC 10^{-6} viable leucocytes, respectively). The administration of CY with the first SRBC injection or two days after however, appeared to have significantly suppressed the memory RFC levels (in comparison with the mean RFC level in toadlets not treated with the drug, group 3, 4 and 1 in Table 7 respectively; group 3 $<$ group 1 $p<0.01$ and group 4 $<$ group 1 $p<0.01$).

(ii) Toadlets first injected with SRBC at a late larval stage (Stage 56):

The results from these experiments are given in Table 8 and are summarized and illustrated in Figure 8 . When given with the first injection of antigen at Stage 56 (Nieuwkoop and Faber, 1967), CY appeared

Group Number	Time of CY injection in relation to the Priming SRBC injection	Splenic RFC 10^{-6} viable leucocytes	
		ICA on day 4	ICA on day 8
1	none	263,444,2220,0	839,11565,1268, 3250
2	-2 days	0,1260	521,191
3	day 0	1270,0	7810,827
4	+2 days	563,3220	5820,0

Table 6 The effect of cyclophosphamide (CY) (150μg.g body weight^{-1}) on the secondary splenic rosette forming cell (RFC) response in Xenopus laevis tadpoles to sheep erythrocytes (SRBC) (3.11 x 10^{8} cells.g body weight^{-1}) when injected at different times in relation to the priming antigen injection.

Rosettes were counted in immunocytoadherence assays (ICA) performed 4 or 8 days after the challenge SRBC injection. For each assay a pool of spleens from 5-7 tadpoles were used. These results are from experiments 1, 2 and 3 in Figure 4.

Group Number	Time of CY injection in relation to the priming SRBC injection	Splenic RFC/10^6 viable leucocytes ($x10^3$)
1	none	1.9*,2.5*,4.4,5.0*,5.6*, 9.5,21.5,25.4*,27.7,31.0, 34.3*,40.3*,58.9,61.3, 62.1,65.1,89.6
2	CY (-2 days)	4.2,4.8,9.1,11.8,15.6, 46.2,47.2,58.4,64.4*
3	CY (day 0)	0.3,0.4,0.8,2.8,4.2,5.5
4	CY (+2 days)	0.3,0.4,0.5,4.0,12.7,17.8

Table 7 The effect of cyclophosphamide (CY) (300μg.g body weight^{-1}) on the secondary splenic rosette forming cell (RFC) response in Xenopus laevis toadlets to sheep erythrocytes (SRBC) when injected at different times in relation to the priming antigen injection. The animals were injected with the primary and a second SRBC injection (3.11 x 10^8 SRBC.g body weight^{-1}, each injection) at larval Stages 48 and 54 respectively. The challenge SRBC injection was given after the end of metamorphosis. These results are from experiments 4, 5 and 6 in Figure 4. *Pools of spleens from two animals were used in the immunocytoadherence assay.

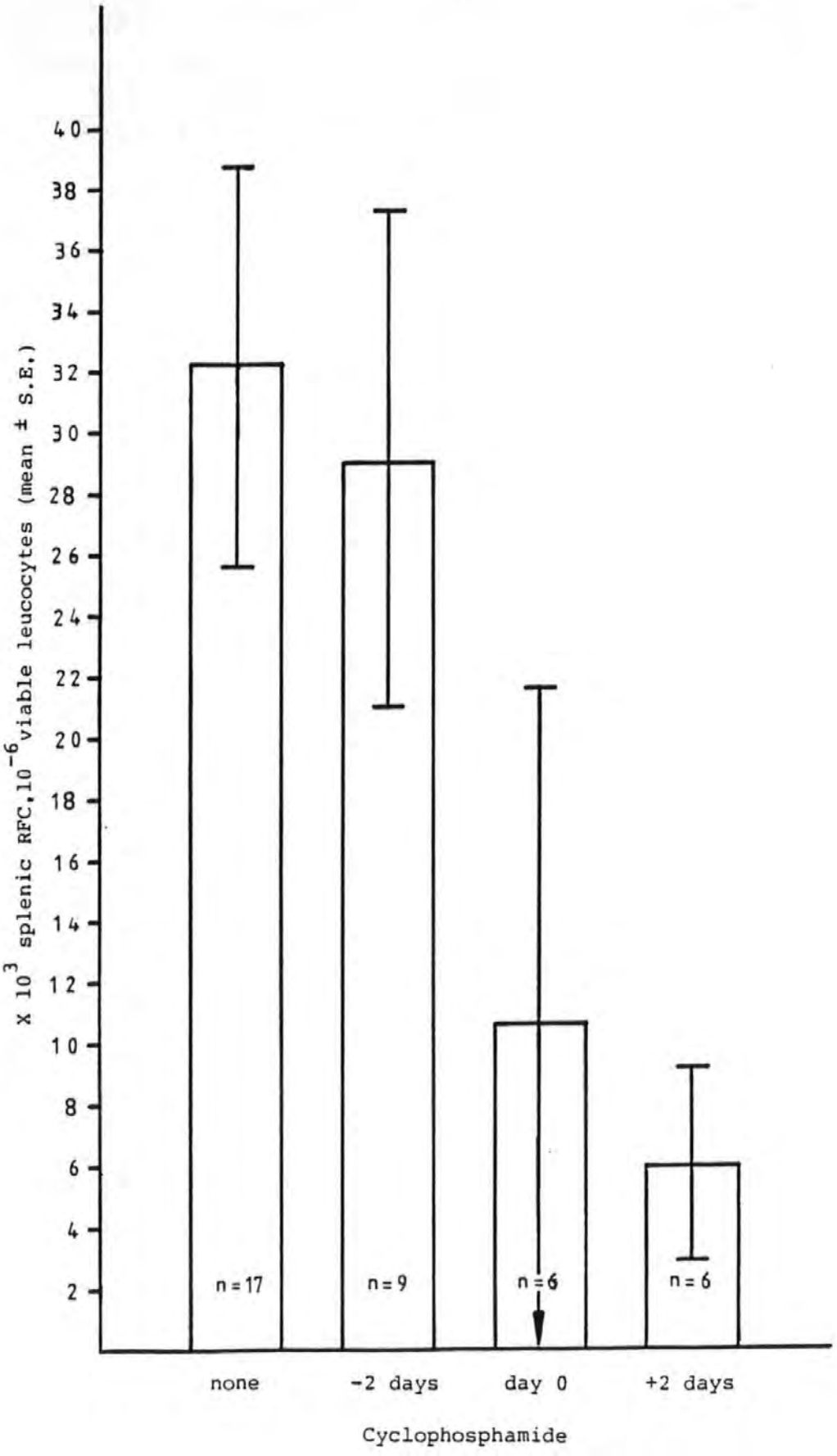
Statistics (U Test)

Group 2 ~ group 1 not significantly different

Group 3 < group 1 $\rho < 0.01$

Group 4 < group 1 $\rho < 0.01$

Figure 7 The effect of cyclophosphamide (CY) (300μg.g body weight^{-1}) on the secondary splenic rosette forming cell (RFC) response in Xenopus laevis toadlets to sheep erythrocytes (SRBC) when injected at different times in relation to the priming antigen injection. The animals were injected with the primary and second SRBC injections (3.11 x 10^8 cells.g body weight^{-1}, each injection) at larval Stages 48 and 54 respectively. The challenge SRBC injection was given after the end of metamorphosis. These results are from Experiments 4, 5 and 6 in Figure 4.
(n) number of animals in each group.


X 10^3 splenic RFC, 10^{-6} viable leucocytes (mean ± S.E.)
40
38
36
34
32
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
n = 17
n = 9
n = 6
n = 6
none
-2 days
day 0
+2 days
Cyclophosphamide

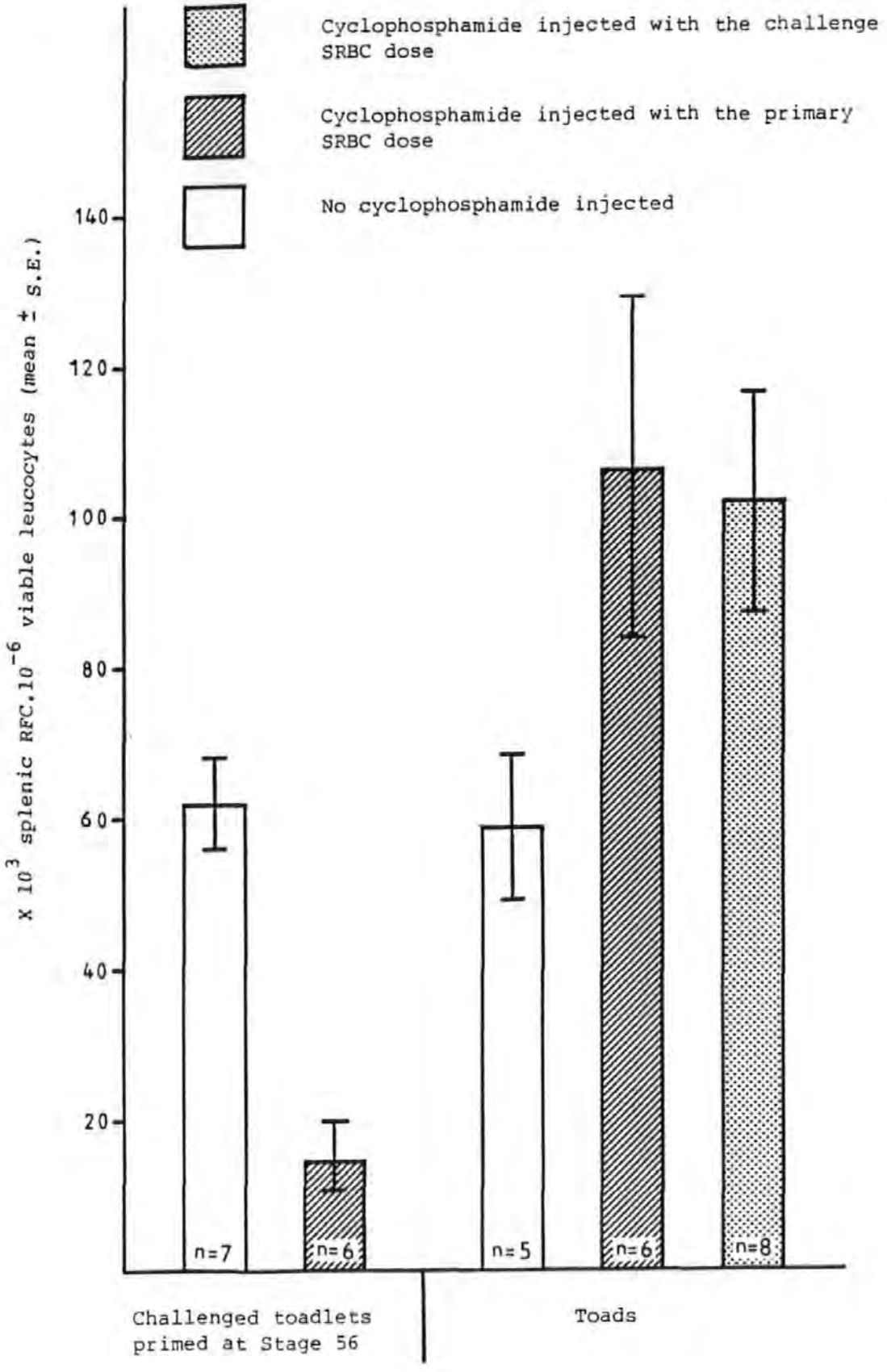
Group Number	Developmental stage at the time of Priming	Challenge	Cyclophosphamide given with Priming Injection	Challenge Injection	$x10^3$ RFC $.10^{-6}$ viable leucocytes	Serum haemagglutinating Antibody titre ($-\log_2$) Individual readings	Mean ± S.E.
1	56	toadlet	none	none	41.9,48.3,49.5,61.3, 67.2,74.3,92.9	not done	not done
2	56	toadlet	yes	none	1.5,4.7,10.6,13.3, 21.8,37.1	not done	not done
3	toad	toad	none	none	35.4,51.3,52.1,66.0, 90.9	4,5,6,7,7	5.8 ± 0.58
4	toad	toad	yes	none	52.2,59.2,84.7,111.8, 125.4,203.6	8,10,10, 10,11,12	10.2 ± 0.55
5	toad	toad	none	yes	60.2,69.0,75.1,82.2, 96.5,106.4,144.7,180.7	not done	not done

Table 8 The effect of cyclophosphamide (CY) (300µg.g body weight^{-1}) on the secondary splenic rosette forming cell (RFC) and serum haemagglutinating antibody titre responses in Xenopus laevis toadlets and toads to sheep erythrocytes (SRBC) (3.11×10^8 cells.g body weight^{-1}) when injected with the priming or challenge antigen injections. These results are from experiments 7, 10 and 11 in Figure 4.

Statistics (U Test)

Group 2 < group 1 $\rho < 0.01$, Group 4 > group 3 $\rho < 0.05$, Group 5 > group 3 $\rho < 0.05$.

Figure 8 The effect of cyclophosphamide (CY) (300µg.g body weight^{-1}) on the secondary splenic rosette forming cell (RFC) response in Xenopus laevis toadlets and toads to sheep erythrocytes (SRBC) (3.11 x 10^8 cells. g body weight^{-1}) when injected with the priming or challenge antigen injections. These results are from Experiments 7, 10 and 11 in Figure 4.
(n) number of animals in each group.


Cyclophosphamide injected with the challenge SRBC dose
Cyclophosphamide injected with the primary SRBC dose
No cyclophosphamide injected
x 10^3 splenic RFC.10^-6 viable leucocytes (mean ± S.E.)
140
120
100
80
60
40
20
n=7
n=6
n=5
n=6
n=8
Challenged toadlets primed at Stage 56
Toads

to have suppressed the memory RFC response of toadlets to SRBC. This response was 76.2% of that obtained from toadlets which had been first immunized with SRBC at Stage 56 but had not received any CY injections (groups 2 and 1 in Table 8 respectively, group 2 $<$ group 1 $\rho<0.01$).

C. RFC memory response in toads treated with CY:-

The data concerning the effect of giving CY with either the first or second (challenge) SRBC injections on the memory RFC and serum antibody responses of Xenopus laevis toads are given in Table 8 and are summarized and illustrated in Figure 8 . In contrast with its suppressive effect on the RFC memory response of toadlets to SRBC, CY appeared to have significantly boosted both the RFC and antibody memory responses in toads to the same antigen ($\rho<0.05$ when CY was given with the second SRBC injection; group 5 in Table 8.

4.4 DISCUSSION

In the present study, cyclophosphamide (CY) does not appear to interfere with the metamorphosis of Xenopus laevis tadpoles. This is in contrast to observations by Balakhnov and Aziz (1979) who found that CY caused some retardation of the metamorphosis of Xenopus laevis tadpoles. The experiments of Balakhnov and Aziz (1979) involved bathing the tadpoles in a solution of the drug at Stage 51 of their development (see Nieuwkoop and Faber, 1967, for staging of Xenopus laevis development) for two hours in a three day course. In the present study, tadpoles injected with CY passed through the stages of metamorphosis at a similar

rate to that of untreated animals. This difference may be partly due to the methods of CY treatment. Gras and Tillo (1981) reported that CY injected into mice does not remain in the circulation for long, (i.e. little more than one hour [Shand, 1979]). This may suggest that a single dose of CY may have had little chance to cause any noticeable effect on the relatively slow metabolic activity of the endocrine glands which are thought to control the process of metamorphosis (Dodd and Dodd, 1976; Gilbert and Freiden, 1981).

The temporal relationship between the injection of SRBC and the injection of CY appear to affect the resulting humoral memory response in adult Xenopus laevis toads. When CY was injected three days after primary or challenge SRBC injections, the RFC and antibody responses were suppressed. In contrast, when CY was injected together with the SRBC injection, memory responses were observed to exceed the normal secondary responses. This may be attributed to the type of cell which was proliferating at the time of CY administration.

The results in this chapter showed that, depending on the dose injected, cyclophosphamide (CY) is potently immunosuppressive to the primary humoral response of Xenopus laevis toads when given three days after the administration of sheep erythrocytes (SRBC). Other experiments indicate that this effect may be due to the elimination or abrogation of the responsive cells (see Table 4). The secondary response was also depressed when CY was given three days after the second (challenge) antigen injection. In the more phylogenetically advanced anuran Rana pipiens, Bugbee et al. (1983) have recorded similar observations of immunosuppression by CY. These workers reported that when measured by immunocytoadherence and haemagglutination assays, the humoral responses

of Rana frogs to the hapten trinitrophenyl (TNP) conjugated to lipopolysacharide of E. Coli (LPS) were suppressed when CY (300mg.kg^{-1} body weight) was given two days after immunization. Bugbee et al. (1983) suggested that CY acts by restricting clonal expansion and also by restricting the synthesis and/or release of antibody but not by generalised lysis of antibody producing cells. Their suggestions regarding the immunosuppressive action of CY however, do not seem to entirely agree with either the results reported here for Xenopus laevis (Table 4) or those reported for higher vertebrates, where CY was also found to cause the depletion of lymphoid cell numbers (Turk, 1967; Turk and Poulter, 1972; Poulter and Turk, 1972; Bach, 1975; Chung et al., 1978; Kim and Ha, 1979; Shand, 1979; Olah et al., 1979; International Agency for Research on Cancer (Lyon) IARC monographs, 1981).

The experiments in the present chapter appear to support observations discussed in Chapter 3. Experiments in the previous chapter (Chapter 3) showed that tadpoles which were given an injection of SRBC at Stage 48 and challenged with the same antigen at Stage 54 did not respond anamnestically when tested by the immunocytoadherence assay. Other experiments in the same study showed that memory however, was generated in those animals since they showed secondary rosette forming cell (RFC) response to the antigen when challenged after metamorphosis as toadlets. Nevertheless, it was not clear whether this memory was initiated after the first or second SRBC injections at Stage 48 and 54, respectively. Toadlets primed at Stage 56 only have also shown a secondary RFC response to a post-metamorphic challenge with the antigen (SRBC). The results of the experiments in the present chapter appear to suggest that the absence of a secondary response during the larval period of Xenopus laevis may have been due to the immaturity of the memory

mechanism in the expression of memory rather than in the generation of memory, assuming that both primary and secondary RFC's belong to one clone of cells. The administration of CY with the first SRBC injection at Stage 48 did not alter the response after challenge at Stage 56. Furthermore, such tadpoles left to metamorphose and challenged as toadlets were unable to raise a secondary RFC response, indicating that unlike control animals not treated with CY, their memory to SRBC had been abrogated. In the light of these observations, therefore, it appears that memory had been induced at Stage 48. This disagrees with previous reports by Kidder et al. (1973) that Xenopus laevis tadpoles at this stage of their development do not respond to immunization with SRBC when tested by the immunocytoadherence assay, since one would expect that the cells responsible for the induced memory would be immunocytoadherence cells. Further analysis in the present study of the timing of CY administration showed that, as in mammals (Botzenhardt and Lemmel, 1975; Sigel et al., 1980; Gras and Tillo, 1981), CY administration into Xenopus laevis tadpoles two days before priming with SRBC had no effect on their secondary RFC response as toadlets, whereas the administration of this drug two days after priming clearly depressed the secondary response. This, therefore, not only implies that CY can be immunosuppressive when injected into Xenopus laevis at an early larval stage (Stage 48) but also suggests that CY has either affected the memory cells themselves, or their precursors at this stage. Interestingly, this argument seems to agree with a recent report by Francus and Siskind (1982) which suggests that during ontogenetic development in mice, the ability to generate memory preceeds the production of antibody secreting cells.

In contrast to the effect of CY on the immune response of mammals to SRBC (Aisenberg and Davis, 1968; Dietrich and Dukor, 1969; Many and

Schwartz, 1970; Marbrooke and Baguley, 1976) CY when given to adult toads with either the priming or challenging SRBC injections in the present study neither led to the induction of tolerance nor did it suppress the response. Instead, humoral responses, haemagglutinating antibody production and RFC production were all augmented. This augmenting effect of CY was however, observed to occur in mammals in humoral responses to antigens other than SRBC (e.g. Bacteriophage fd and BSA, Bruner et al., 1981; Pneumoncoccal polysaccharide type III, Bagsra et al., 1981) as well as in cell medicated responses (Turk, 1973; Polak, 1977; Asherson and Zembala, 1978; Corrier et al., 1979). Some authors relate this effect to an "over-shoot" in the recovery of depleted lymphocytes (Chung et al., 1978; Mazigh et al., 1979; Sigel et al., 1980; Gras and Tillo, 1981). Other authors however, relate such enhancement of the immune response to the inhibition of the differentiation and activity of suppressor cells (Zembala and Asherson, 1976; Polak, 1977; Asherson and Zembala, 1978; Batchelor, 1979; Bagsra et al., 1981; Bruner et al., 1981; Bwyer et al., 1981; Ozer et al., 1982).

The role of such suppressor cells in the immune response of mammals is a regulatory one (Gershon, 1978). They were found to be involved in the control of T and B cell activities (Dutton and Swain, 1982). It has recently been reported that the anuran amphibian Xenopus laevis may have such cells in their immune system (Du Pasquier and Bernard, 1980; Du Pasquier, 1982). It is possible, therefore, that in the present study CY may have depleted to some extent a population of suppressor cells, allowing the clone of responding cells to expand. Moreover, there is some experimental evidence to suggest that such suppressor cells in Xenopus laevis may be T (thymus) cells (Gruenwald and Ruben, 1979; Ruben et al., 1980). These authors found that adult thymectomy in

Xenopus laevis led to an enhanced RFC response to foreign erythrocytes.

The assumption that the administration of CY depletes a population of suppressor cells seems to explain both the augmented responses and the "over-shoot" in recovery of cell numbers. This becomes clearer when the mechanisms by which suppressor cells interact with target cells are considered. The suppressor cells produce soluble products or factors which have lymphokine properties (Dutton and Swain, 1982). Some of these products suppress the clonal expansion of target lymphocytes. Conversely, their absence or the absence of their sources would enhance clonal expansion of target lymphocytes.

In summary, a regulatory suppressor mechanism does not appear to have developed in Xenopus laevis tadpoles. Such a mechanism however, appears to have developed either during or after metamorphosis. In tadpoles, memory cells may not differentiate from the same population of cells producing the RFC since CY abrogates the secondary RFC response while a primary level is maintained.

It is clear from the above results and discussion that the difference in the effect of CY on memory RFC response to SRBC in tadpoles and adults of Xenopus laevis denotes or underlines, to some extent, a difference in the regulatory mechanism(s). The indications appear to be that in tadpoles the regulatory mechanism may have a predominantly helper function, whilst in adults a suppressor function may prevail. This may be attributed to the state of differentiation and maturity of the regulatory mechanism in the immune system of Xenopus laevis.

- C H A P T E R F I V E -

- C H A P T E R F I V E -

TRANSPLANTATION MEMORY IN XENOPUS LAEVIS TO ALLOGENEIC BLOOD LEUCOCYTES INJECTED DURING ONTOGENY

5.1 INTRODUCTION

One of the most stimulating questions in transplantation immunology was asked by Ehrlich and Morgenroth (1900) who wondered why animals which respond to a great number of antigens including tissues, do not equally recognise and respond to their own "antigens". The inspiration for a model which could resolve this problem experimentally came from the observations of Owen (1945); he found that anastomotic cattle twins (Owen, 1945, 1946) and twins resulting from superfecundation (Owen, 1945) were blood chimaerae. These observations led Burnet and Fenner (1949) to postulate that animals can be rendered tolerant to foreign antigens by exposure to these antigens during the early developmental stages. It was not long however, before this theory was put to the test by the pioneering work of Billingham, Brent and Medawar (1953). They were the first to report the experimental induction of transplantation tolerance in mice and chickens. They found that host animals which had been exposed at an immature stage of development to cells from allogeneic donors will tolerate skin grafts from these donors when these are applied later in life. Since then scientists have devised many other methods for the induction of transplantation tolerance (see Steinmuller, 1979). The main objective however, is still to gain an understanding of the mechanisms of "self" recognition and tolerance.

The methods of tolerance induction to tranplantation antigens

include the joining of embryonic circulatory systems (parabiosis or anastomosis), the aggregation of embryonic blastomeres, the inoculation of cells or the application of tissue grafts during immature stages of development, the reconstitution of irradiated adults with allogeneic bone marrow cells and the use of immunosuppressive agents, such as alkylating drugs or anti-lymphocyte sera used together with the allogeneic antigens (either in the form of whole cells or as homogenates). The success rate, in terms of the number of resulting tolerant animals from the total number of animals treated, is not necessarily the same in all the cases. Amongst the best established of these methods is the administration of allogeneic cells into foetal, embryonic or neonatal hosts (Billingham et al., 1953, 1955, 1956; Woodruff and Simpson, 1955, Billingham and Brent, 1956, 1957).

In mammals, the tolerant animals obtained by the infusion of allogeneic cells into newborn recipients are chimaerae, but whether their chimaerism is a cause or effect of their tolerant state is not certain. Futhermore, the outcome of such treatment in terms of operative tolerance depends to some extent on the type and dose of cells infused, the route of their administration and the state of maturation of the host.

It seems that various circumstances and different levels of complete or partial tolerance may lead to different sub-populations of lymphocytes becoming tolerized and attempts have been made to analyse the conditions which may lead to clonal deletion or to "active" suppression (Beverley et al., 1973; Rouse and Warner, 1974; Von Boehmer et al., 1975; Wright et al., 1975; Brooks, 1975; Kindred and Sordat, 1977; Batchelor 1979; Roser and Dorsch, 1979; Vegh et al., 1980;

Streilein and Gruchalla, 1981; Tutschka et al., 1981). For example, using neonatal mice, Brooks found that fully tolerant animals showed no mixed leucocyte reactions or cytotoxic cells in in vitro tests whereas partially tolerant animals often possessed cells which responded in mixed leucocyte cultures, this effect being related to the dose of cells administered. Mammalian experiments of this nature are essentially performed on relatively late stages of development (by amphibian standards) or are performed on irradiated hosts. Much earlier exposure to allogeneic tissue can be achieved in mammals by the use of allophenic (tetraparental) mice (e.g. Phillips and Wegmann, 1973; Von Boehmer et al., 1975).

In spite of the successful studies on embryonically or foetally induced transplantation tolerance in birds and mammals, the fact that the immature stages are not easily accessible for experimental manipulation in vivo and/or are under some maternal influence remains an obstacle. Amphibians however, characterized by their free living and easily manipulatable larvae, offer a possible model for studies on the early induction of transplantation tolerance. Such studies have mostly been conducted on anuran amphibians in general and on Xenopus laevis species in particular (see Volpe, 1970; Clark and Newth, 1972; Chardonnens and Du Pasquier, 1973; Cooper, 1973, 1976; Du Pasquier, 1973, 1976, 1982; Du Pasquier and Chardonnens, 1975; DiMarzo and Cohen, 1979; Manning and Botham, 1979; Sala and Cardellini, 1979; Botham and Manning, 1980a, 1980b; Cohen et al., 1980; Barlow et al., 1981).

Some of the methods used to induce transplantation tolerance in anurans have involved the transfer of relatively large flank grafts

from embryo to embryo (Clark and Newth, 1972) or skin grafts from adults to tadpoles (Chardonnens and Du Pasquier, 1973; DiMarzo and Cohen, 1979). Parabiosis or the joining of embryos is another method used to induce tolerance in anurans, not only between allogeneic combinations (Huchon, 1962; Davison, 1966; Volpe and Gebhardt, 1968) but also between xenogeneic combinations (Sala and Cardellini, 1979), although in the latter case tolerant tadpoles almost never reached metamorphosis.

Grafts of flank tissue can be exchanged between 48 hour post-fertilization Xenopus laevis embryos resulting in animals which are tolerant of the partner's skin (Clark and Newth, 1972) and which are unreactive against their partner when their spleen cells are reacted together in mixed lymphocyte reactions (Manning and Botham, 1979; Botham and Manning, 1980a, 1980b). These animals were thought to be chimaeric. This would be consonant with a series of experiments designed to study the origin of anuran lymphocytes which were conducted using a similar method in both Xenopus and Rana species and in which chimaerism was demonstrated (Tompkins et al., 1979; Volpe et al., 1979; Tompkins et al., 1980).

Xenopus laevis can, in fact, be rendered tolerant throughout the entire larval life (especially during the period of metamorphosis) to the antigenic products of both major and minor histocompatibility loci, the outcome depending on factors such as maturity, MHC disparity, gene dose and the amount of donor skin tissue grafted (Chardonnens and Du Pasquier, 1973; Chardonnens, 1975; Du Pasquier and Chardonnens, 1975; Barlow and Cohen, 1979; DiMarzo and Cohen, 1979; Cohen et al., 1980; Barlow et al., 1981). Futhermore, there is some experimental evidence

that this type of tolerance may involve active suppression by a population of suppressor cells (Du Pasquier and Bernard, 1980; Du Pasquier, 1982).

There are at least two reports on successfully induced tolerance to skin grafts from Rana temporaria in allogeneic hosts and xenogeneic hosts as well (Rana arialis) achieved by infusing the hosts at a larval stage with skin cell homogenates from the donors (Vyazov and Sorokina, 1961, 1962).

Further work using this method has not appeared in the literature, yet it is clear that such a method would provide an easy tool for the performance of more precise investigations on the mechanisms of induction and maintenance of transplantation tolerance in anurans; more precise assessment of the type and number of donor cells as well as the most efficient route of their administration for the induction and maintenance of tolerance would be possible.

In the present chapter attempts to induce either tolerance or positive anamnestic graft rejection to allogeneic skin grafts by the injection of allogeneic donor peripheral blood leucocytes in Xenopus laevis will be described. The host's state of differentiation, the number of donor cells and the route of their administration are investigated as possible parameters influencing the resulting memory, the assessment of which was monitored by means of allograft rejection experiments and mixed leucocyte reactions in vitro.

5.2. MATERIALS AND METHODS:

5.2.1 Animals injected as tadpoles:

The protocol for these experiments is shown in Figures 9 and 10. Adult allogeneic peripheral blood leucocyte suspensions (ABL) were prepared following the method described below. The cells were injected into groups of tadpoles (leucocyte recipients [LR]) representing different stages of development from Stage 47 to Stage 57 (Nieuwkoop and Faber, 1967). Each tadpole received a dose of the allogeneic blood leucocytes which varied from 1×10^4 cells per tadpole to 9×10^5 cells per tadpole, delivered in a volume of 3-10µl of fluid. The cells were injected intraperitoneally (i.p.), intramuscularly (i.m.), intrathymically (i.t.) or into the circulation (i.c.) by the intracardiac or intravenous route (see Chapter 2). Control animals were either injected at similar stages of development using the same routes with corresponding volumes of fluid only (the injected controls I.C.) or did not receive any injection (normal controls, N.C.).

All animals were reared in separate tanks of standing (de-chlorinated) tap water at room temperature ($18 \pm 1^{\circ}C$) as described in Chapter 2. At 2 - 6 months post-metamorphosis the toadlets were divided into two groups, one of which was used in mixed leucocyte cultures (MLC), the other for skin graft rejection experiments (see below). In the mixed leucocyte cultures, spleen cells from each of the leucocyte recipient (LR) and control (IC and NC) toadlets were tested in two-way MLC against spleen cells from their leucocyte donor and from "third party" unrelated adult controls (CD). In the skin graft experiments each animal (leucocyte recipients [LR]) and controls (IC and NC) received skin allografts from the corresponding donor (LD), from a "third party" donor

Figure 9 Experimental protocol for studying the effect of allogeneic adult blood leucocytes (ABL) on allograft rejection (G) and spleen cell reactivity in mixed leucocyte cultures (MLC) in Xenopus laevis toadlets.

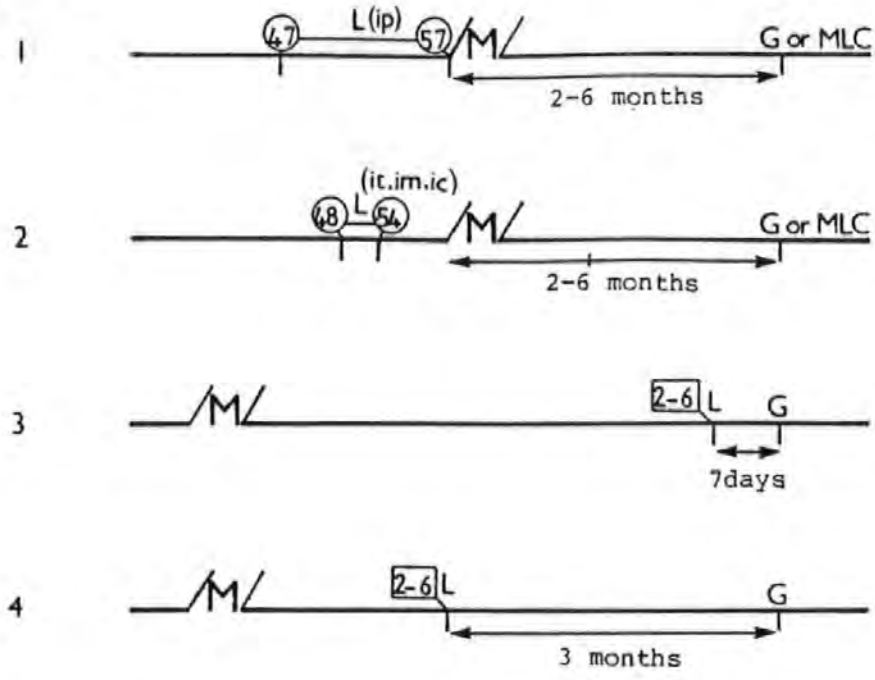
1 The effect of ABL injected intraperitoneally at various larval stages

2 The effect of ABL injected by various routes during the larval period

3 The effect of ABL injected intraperitoneally into toadlets one week before grafting

4 The effect of ABL injected intraperitoneally into toadlets three months before grafting.

Experiment No:



Key:

: Allogeneic adult blood leucocytes injection. (ip)(it,im,ic) intraperitoneal, intrathymic, intramuscular and intracirculation routes of injection.

: Skin graft rejection reactivity.

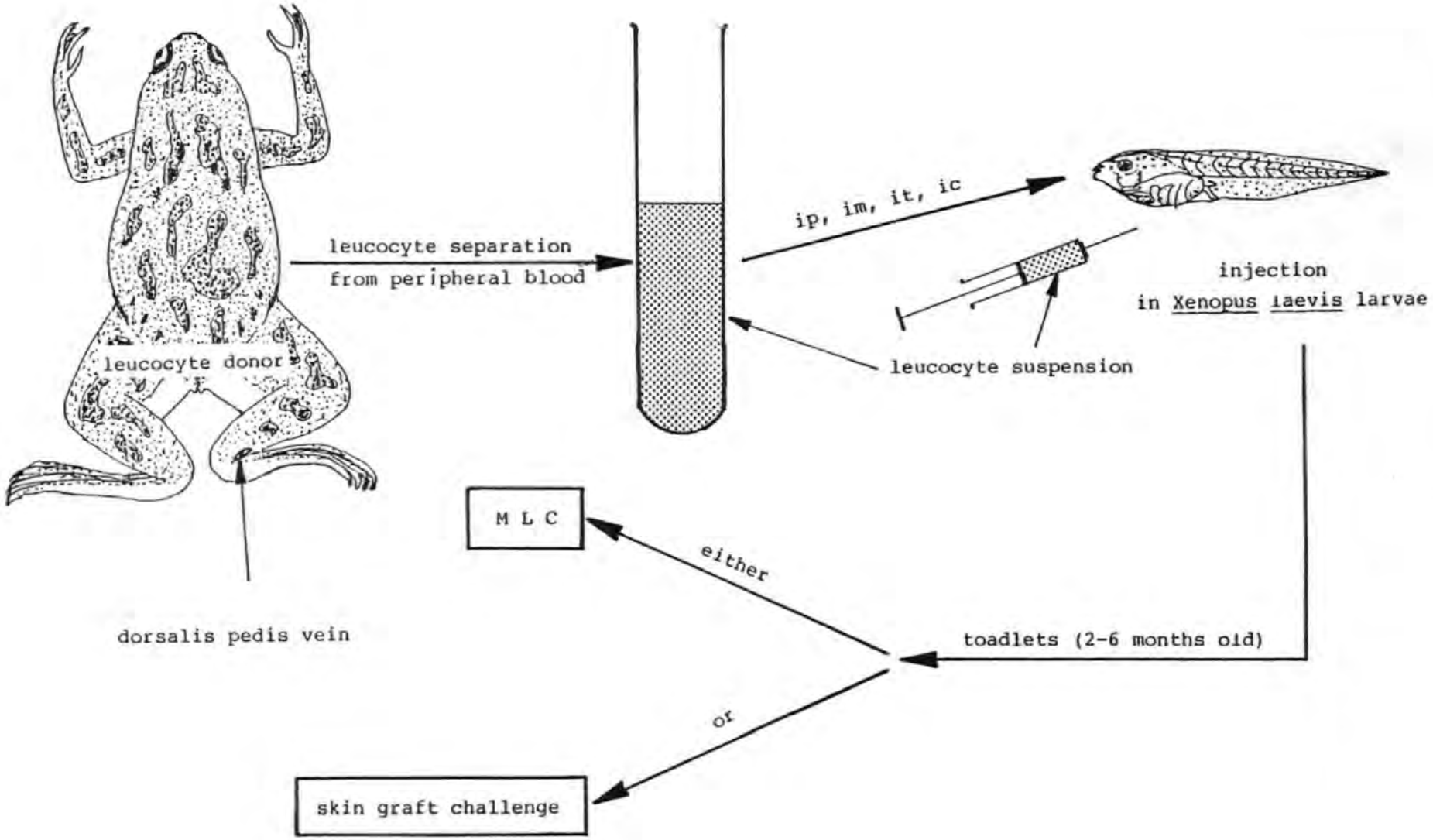
: Mixed leucocyte culture.

: Metamorphosis.

47,48, 54,57 : Larval stages (Nieuwkoop and Faber, 1967).

2-6 : Age of post-metamorphic toadlets in months.

Figure 10 Diagramatic representation of the experimental protocol used to study the effect of allogeneic adult leucocytes (ABL) injected during the larval period on allograft rejection and spleen cell reactivity in mixed leucocyte cultures (MLC) in Xenopus laevis toadlets.


leucocyte donor
dorsalis pedis vein
leucocyte separation
from peripheral blood
ip, im, it, ic
injection
in Xenopus laevis larvae
leucocyte suspension
M L C
either
toadlets (2-6 months old)
or
skin graft challenge

(CD) and from itself (an autograft) (Figure 11).

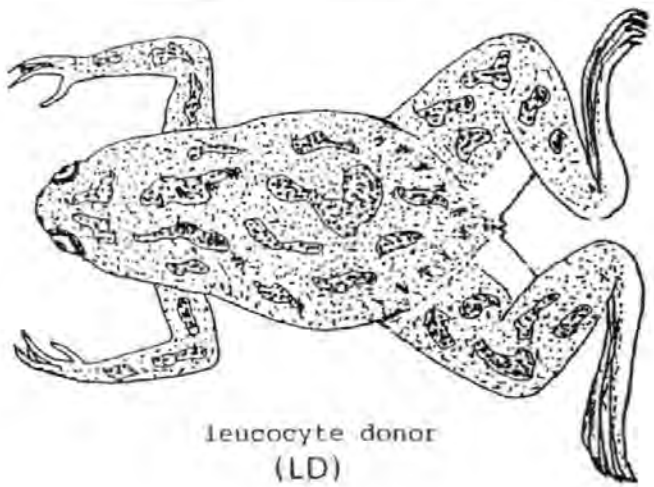
5.2.2 Animals injected as 2-6 month old toadlets:

Eight 2-6 month old toadlets were injected intraperitoneally with a single dose of 2×10^7 allogeneic blood leucocytes. They were then divided into two groups and grafted with skin from their corresponding donor (LD), from a "third party" donor (CD) and from themselves; the first group being grafted at one week and the second group at three months after receiving the injection of allogeneic cells. Non injected controls (NC) were also grafted with similar grafts on the same day (see Figure 9 and Figure 11). Injected controls (IC) were not used in these experiments.

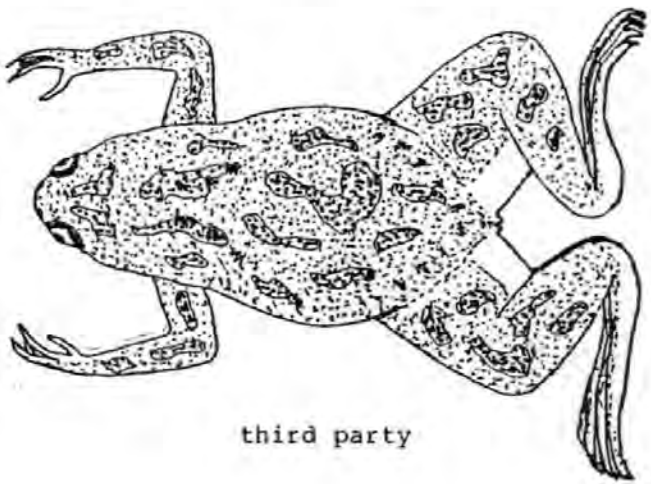
5.2.3 Preparation and injection of the allogeneic leucocytes:

Under sterile conditions, the blood was collected from the dorsalis pedis vein (see Millard, 1941) of adult leucocyte donors using heparinised pasteur pipettes, 1 cm^3 of blood was diluted in 1.5cm^3 of heparinised Lebovitz-L15 medium (Gibco, Biocult) diluted with distilled water. This was layered on 4 cm^3 of lymphocyte separation medium (Flow Laboratories, Irvine) in a 10 cm^3 centrifuge tube. The tube was centrifuged for 10-12 minutes at 800 rpm. The leucocytes, mainly consisting of lymphocytes, were collected from the cloudy layer above the separation medium. They were washed twice and resuspended in diluted L15 medium and counted. The yield of leucocytes was $9 \times 10^6 \pm 1 \times 10^6$ cells cm^{-3} of blood, with $1 \times 10^5 \pm 1 \times 10^4$ RBC's contamination. The leucocytes were injected intraperitoneally into the tadpoles larvae (leucocyte recipient) using No. 30 gauge needles.

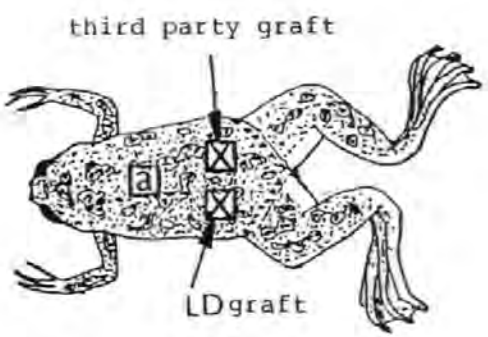
Figure 11- Skin grafting experiment,(a)autograft, (⊠)graft rejected.



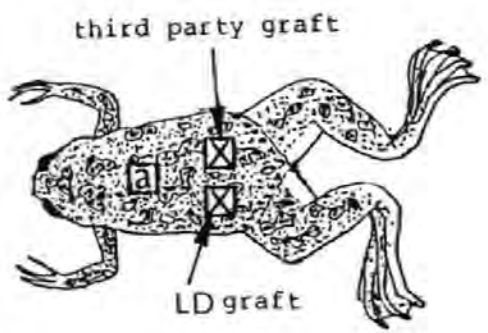
leucocyte donor
(LD)



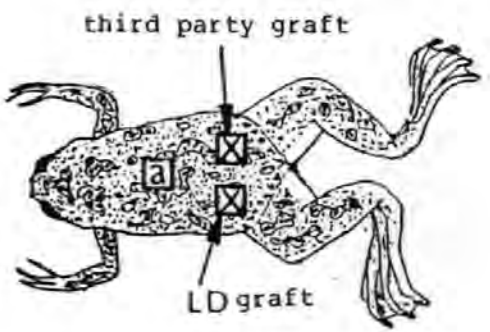
third party



leucocyte injected host



medium injected control



non injected control

Control animals were injected with 2 to 10cm^3 of diluted L15 medium.

5.2.4 Mixed leucocyte cultures:

All experiments were carried out under aseptic conditions until the time when the cells were harvested. Animals were sacrificed in MS222 (2g MS222 in 400 cm^3 of water). Spleen cell suspensions were prepared from their teased spleens, washed twice and suspended at a concentration of 1 x 10^6 cells cm^{-3} in culture medium.

For control cultures, 0.2 cm^3 of one population of cells was dispensed in one well of a 96 well microtitre plate. For experimental cultures, 0.1 cm^3 of one cell suspension was mixed with 0.1 cm^3 of a second cell suspension. All control and experimental cultures were run in five wells for each culture. The culture plates were placed in an air-tight culture cabinet, filled with an atmosphere of 5% CO_2 in 95% air and incubated for 5 days at 26^0C. On the fourth day of incubation the cultures were pulsed with 2mCi. of ^{3}(H)-thymidine (Radiochemical Centre, Amersham, Bucks.) per well. On the fifth day of culture, the cultures were harvested in a Titretek cell harvester (Flow Laboratories, Scotland) by washing with distilled water on glass fiber filter papers. The cells harvested from one culture well were placed in a polythene vial and solubilized by incubation with 0.3 cm^3 Protosol per vial (New England Nuclear). In each vial, 5 cm^3 of PPOP/POPOP toluene solution (Scintiprep, Fisher) or 5 cm^3 of Fisoflour-2 (Fison) were added and the counts/minute (c.p.m.) of the β-emissions produced from the radioactive material in the vials were counted in a liquid scintillation counter. Stimulation indices (S.I.) were

calculated from the ratio of the c.p.m. from the mixed cultures divided by the mean count of the control cultures

$$\text{i.e.} \quad S.I. = \frac{\text{c.p.m. A + B}}{0.5 \times (\text{c.p.m. A} + \text{c.p.m. B})}$$

5.2.5 Skin grafting:

These experiments were performed following the technique described by Horton (1969) for Xenopus laevis. The animals used were always post-metamorphic toadlets and toads. The host and donor animals were anaesthetized in 1.5 : 1000 (w/v) solution of tricaine methane sulphonate MS222 (Sigma St. Louis or Sandoz Basel) and placed, with their dorsal sides facing upwards, on a wet bed of cotton wool in a glass tray under a dissection microscope. Three $2mm^2$ graft beds were prepared in the dorsal skin of each host by a sharp fine pair of scissors. One graft bed received a self skin graft (autograft), the others received allogeneic skin grafts from the donors. The hosts were left for 15 minutes outside the water, allowing the skin grafts to stick in their places, then the animals were replaced in clean standing tap water and left in a quiet dark room to recover without vigorous movements of the sort which might dislodge their grafts.

The hosts were inspected every three days for signs of skin graft rejection. The grafts were considered to be rejected when most of the pigmentation had disappeared and only collagenous dermal structures remained.

5.3. RESULTS:

5.3.1. Responses in mixed leucocyte cultures:

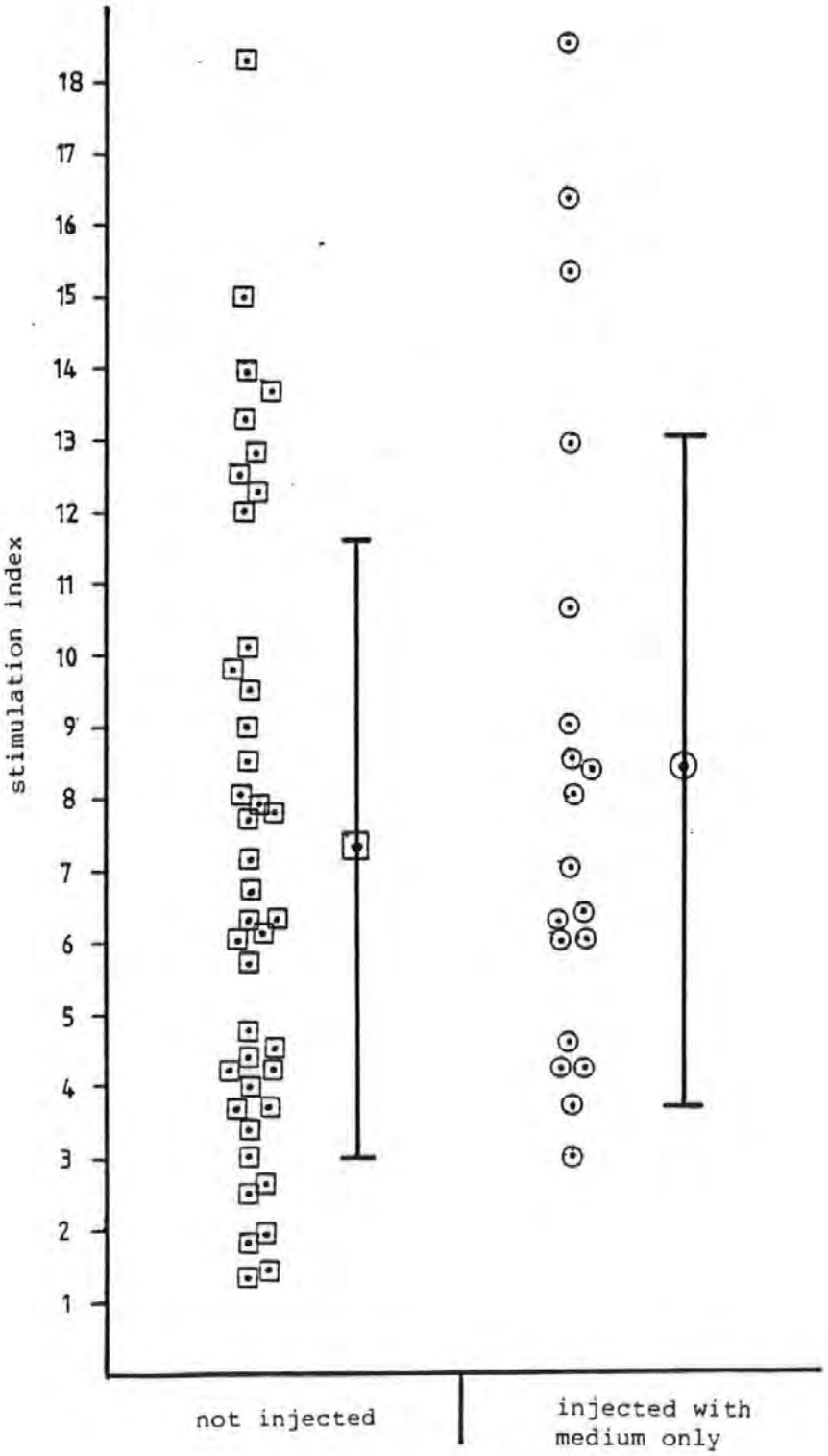
A. Animals injected at different stages of development:

Figure 12 represents the responses obtained from two-way mixed lymphocyte cultures in which spleen cells from 2-6 month post-metamorphic toadlets were reacted against adult toads of the donor series (LD or CD) or against normal controls (NC). The mean stimulation index (SI) obtained from these cultures was 7.31 ± 4.25 (mean $\pm$ standard deviation n-1, n = 41). The high value for the standard deviation may indicate a wide range in the histocompatibility disparities between the tested animals from our stock. The toadlets used included injected controls (IC) as well as normal untreated animals and the results confirm that the injection of medium alone had no effect on the stimulation indices obtained (Figure 12).

Figure 13 shows the results obtained when spleen cells from the recipients of allogeneic leucocytes (LR) were reacted either with spleen cells from their donor (LD) or with "third party" spleen cells (CD). The animals were at larval stages between Stage 47 and Stage 57 when injected with allogeneic leucocytes. They were killed for the mixed leucocyte experiment as 2-6 month post-metamorphic toadlets. From Figure 13 it can be seen that the stimulation indices obtained when leucocyte recipients were reacted against their corresponding leucocyte donors did not differ signigicantly from those obtained in "third party" or normal MLC reactions. Thus an intraperitoneal injection of adult blood leucocytes failed to induce allogeneic transplantation tolerance in these experiments even when administered to *Xenopus laevis* larvae as

Figure 12 Two-Way MLC in Xenopus laevis

MLC stimulation indices obtained when cells from 2-6 month old toadlets were cultured with spleen cells from an adult toad of the donor series or from a normal control. The toadlets were either pre-injected as tadpoles with a diluted solution of L-15 (⊙) or not injected (⊡). Symbols with bars (I) represent the means ± standard deviation.


stimulation index
18
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
not injected
injected with medium only

(NC) (IC)

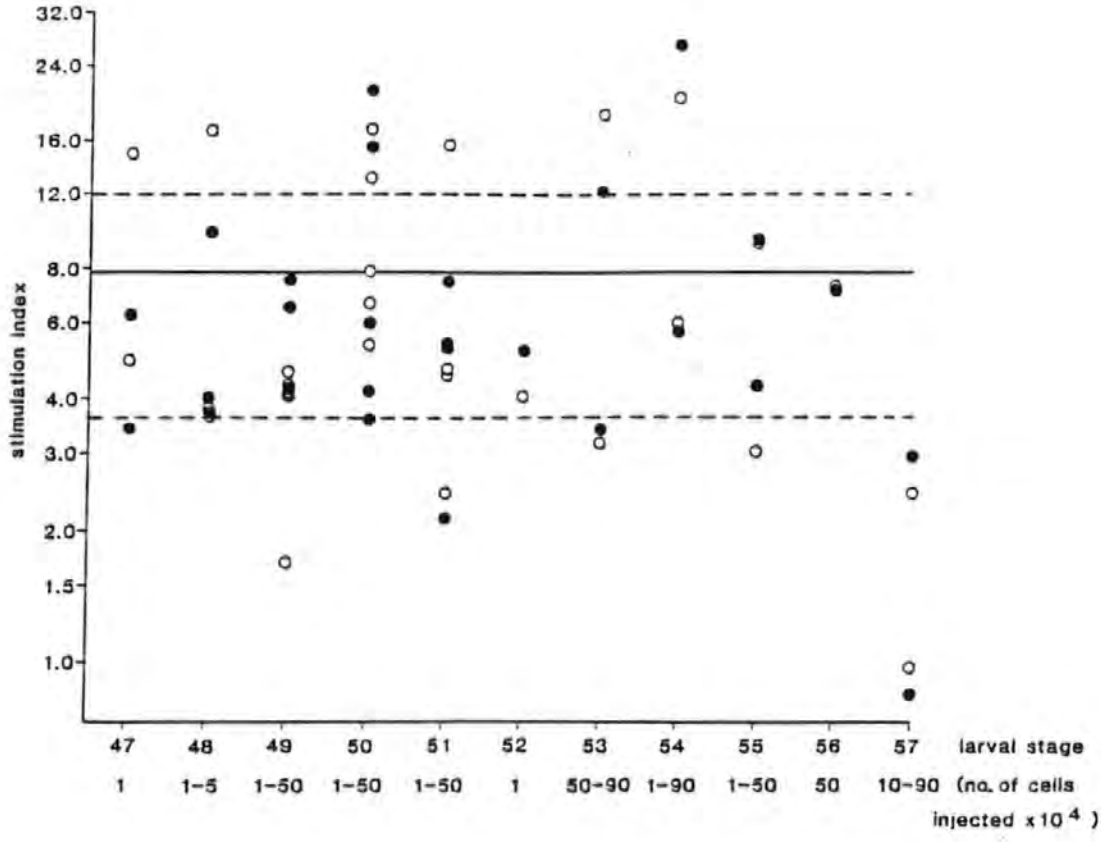


Figure 13 S.I. from two-way MLC from experiment involving ip. injections of Xenopus laevis larvae with allogeneic cells. (●) S.I. from MLC with donor (O) S.I. from MLC with third party. (——) mean and (- - - -) standard deviation of S.I. from normal v. normal control experiments. These results are from Experiment 1 in Figure 9.

early as Stage 47 of their ontogenetic development.

B. The Effect of the Route of injection:

Table 9 shows the results obtained when Xenopus laevis larvae at different stages of development were injected with a single dose of 3-10 µl of a cell suspension containing 0.5 - 1.0 x 10^5 adult blood leucocytes via different routes (intramuscular, intrathymic and via the circulation). They were killed as 2-6 month post-metamorphic toadlets and their spleen cells were tested in mixed leucocyte cultures against spleen cells from the corresponding leucocyte donors and spleen cells from "third party" controls. From Table 9 it can be seen that there is again no evidence that injection of allogeneic cells into young larvae had any effect upon subsequent mixed leucocyte reactions with the donor cells.

5.3.2 Responses to skin allografts:

A. Animals injected as tadpoles with allogeneic blood leucocytes:

The animals were injected with adult blood leucocytes at larval stages between Stage 47 and Stage 54, then grafted as 2-6 month post-metamorphic toadlets.

One week after application, all grafts were infiltrated by blood capillaries which were observed to appear by day 3. The intensity of this infiltration gradually regressed in autografts as they healed. In allografts, on the other hand, whether taken from the leucocyte donor or from a "third party" control, this infiltration intensified

Table 9 Stimulation indices from MLC between toadlets injected with allogeneic adult blood leucocytes (ABL) via different routes. These results are from Experiment 2 in Figure 9.

Developmental Stage at time of injection	Route of Injection	No of Cells injected	S.I. from MLC with Donor	Third Party
48	intrathymic	5×10^4	N.D.	5.4,5.29
48	intrathymic	5×10^4	N.D.	5.3,4.02
50	intrathymic	$0.5\text{-}1 \times 10^5$	N.D.	5.49
51	intrathymic	$0.5\text{-}1 \times 10^5$	N.D.	6.54
52	intrathymic	$0.5\text{-}1 \times 10^5$	2.88	5.66
54	intrathymic		4.43	6.43
no injection	no injection	none	2.58	6.23,7.96 3.78
49	intracirculation	$0.5\text{-}1 \times 10^5$	1.00,1.61	3.04,1.56, 1.09
50	intracirculation	$0.5\text{-}1 \times 10^5$	N.D.	1.24
no injection	no injection	none	1.34	4.45,1.81
50	intramuscular	$1\text{-}5 \times 10^5$	4.9	3.83
53	intramuscular	$1\text{-}5 \times 10^5$	10.83	8.86
54	intramuscular	$1\text{-}5 \times 10^5$	3.15	3.98
no injection	no injection	none	2.59,6.81	6.23,3.97

and eventually led to graft rejection. The rejection times are shown in Table 10, from which it can be seen that injection of allogeneic cells into young larvae did not affect their ability to reject donor skin.

B. Animals injected as toadlets with allogeneic blood leucocytes:

In toadlets, whether grafted 7 days or 90 days after the injection of allogeneic cells, autografts were retained and allografts were rejected, irrespective of whether the allograft came from the leucocyte donor or from a "third party" control. The pattern of allograft rejection was similar to that described above and no differences were observed in the graft survival times between donor and "third party" grafts (see Table 11).

5.4 DISCUSSION:

In the experiments presented above, allogeneic donor peripheral blood leucocytes were injected by various routes (intraperitoneal, intramuscular, intrathymic and into the circulation) into either larval hosts at various pre-metamorphic Stages 47 - 57 of Nieuwkoop and Faber (1967) or post-metamorphic toadlets (2-6 months post-metamorphosis). The hosts injected as tadpoles were tested at the age of 2-6 months post-metamorphosis either with skin allografts or in two-way mixed leucocyte cultures (MLC). Hosts injected as toadlets, on the other hand, were tested only with skin allografts applied one week or three months after receiving the allogeneic cells. The results seem to suggest that neither tolerance nor positive memory have been conferred by such treatment in Xenopus laevis. Other reports (Horton and Horton,

Table 10 Rejection times of skin allografts in Xenopus laevis toadlets, either normal controls or toadlets injected as tadpoles with allogeneic adult blood leucocytes (ABL). The controls include animals injected with medium alone as tadpoles. These results are from Experiments 1 and 2 in Figure 9.

* Experiments carried out at lower temperature ($18^{\circ}C \pm 1$) as compared with the others ($22^{\circ}C \pm 1$).

ip., im., it., ic. : intraperitoneal, intramuscular, intrathymic, intracirculation respectively.

Developmental Stage at time of injection	Route of Injection	No of cells injected	Rejection Times (Days) of Skin grafts on Injected hosts from Donor	Third Party	Skin grafts on Normal controls
47	ip	1×10^4	20*,27*	13+,22*	26*,26*,13*
47	im	$0.1\text{-}1 \times 10^5$	19,19,27*	16,19,28*	19,19
48	ip	5×10^4	22*,22*,22*	22*,22*,22*	26*,26*,26*,26*, 26*,26*
48	im	$0.5\text{-}5 \times 10^5$	22,19,22,19, 16,26	16,19,19,16,16	19,16,26,20
48	it	$0.5\text{-}5 \times 10^5$	27*,28*,26	27*,16	27*,27*,26,20
51	im	$0.5\text{-}5 \times 10^5$	21*,27*,20,21	27*,20,17	20,20,21
51	it	$1\text{-}5 \times 10^5$	22,16	20,16	23,23
51	ic	$1\text{-}5 \times 10^5$	15,15	13,20	23,23
51	ip	$1\text{-}5 \times 10^5$	24*,20*	24*,18*	21*,21*,20*,20*
53	im	$0.5\text{-}5 \times 10^5$	27*,16,27*,27*	21*,16,27*,27*	20,20,27*
53	ip	1.5×10^5	27*,27*,20*	27*,27*,20*	27*,27*,27*,27*, 23*,15*
54	ip	$0.5\text{-}5 \times 10^5$	23*,27*,23*	23*,27*,20*	27*,27*,27*,27*, 23*,15*

Time between cell injection and grafting (days)	Rejection times (days) of Skin grafts on injected hosts from		Skin grafts on normal controls
	Donor	Third party	
7 (*)	23,27,23,26	27,27,26	23,27,27,36,32,26,23
90	20,20,20	24,24,20,20	20,20,20,20,20

Table 11 Rejection times of skin allografts following intraperitoneal injections of allogeneic adult blood leucocytes (ABL) into 2-6 months post-metamorphic Xenopus laevis toadlets. Each animal received one injection of 2×10^7 ABL. (*) Experiments carried out at lower temperature ($18^{\circ}C \pm 1$) as compared with the others ($22^{\circ}C \pm 1$). These results are from Experiments 3 and 4 in Figure 9.

1975; Tochinai et al., 1976; Nagata and Tochinai, 1978; Kawahara et al., 1980; Katagiri et al., 1980; Nagata, 1980) indicate that allogeneic lymphocytes from various tissues including peripheral blood can perform and restore immunological function in Xenopus laevis toadlets thymectomized as larvae. Other workers (Du Pasquier and Bernard, 1980) have found that adult tetraploid (4n = 72 chromosomes) lymphocytes injected into diploid isogeneic tadpoles could be detected both in the spleen and thymus one week after administration. How long these infused cells remain inside the hosts and whether or not the hosts would be tolerant to the donors of these cells is not certain. It is likely however, that such cells would gradually disappear from the hosts' circulation, since exparabiotic animals were reported to lose both their chimaeric status and tolerance to their partners' skin with time (Volpe, 1970, 1971).

Xenopus laevis tadpoles and toads are known to be capable of producing memory response to allografts. It has been shown that tadpoles (Rimmer, 1976; Nagata, 1976) and toads (Simnett, 1965; Horton and Horton, 1975; Rimmer and Horton, 1977) of Xenopus laevis can reject second set allografts in an accelerated way, compared with the rejection rate for first set grafts.

The present results are, however, in agreement with those of Hildemann (1958) from his experiments on adult fish, using whole allogeneic blood infused intraperitoneally. Although the fish could not be primed by the intraperitoneal route, the authors were however, able to induce second set reactivity after priming subcutaneously. From this it seems that the success of conferring tolerance by antigen administration depends to some extent on the

route of injection; some routes were reported to be more effective than others (Billingham and Brent, 1956, 1959; Billingham et al., 1957; Hildemann, 1958; Horiuchi and Wakeman, 1968; Ohara et al., 1979). This however, does not seem to apply in the present case; even when the innoculum was administered directly into the thymus it failed to confer immunological memory, inducing neither positive memory nor tolerance. The intrathymic route has been reported as the most efficient for the induction of tolerance in both mice (Ohara et al., 1979) and rats (Horiuchi and Wakeman, 1968).

It is thought that the present results may also be due to some extent to the inefficiency of the inoculum used, which may be due to the quality or quantity of the cells used. Peripheral blood leucocytes of Xenopus laevis have been shown to be immunologically competent, i.e. they can respond immunologically in MLC (Weiss and Du Pasquier, 1973) and they can induce the production of allo-antibodies in adult toads (Du Pasquier et al., 1979). Viability tests on the cells used in the present experiments have always shown that they were at least 95% viable. This, on the other hand, does not mean that they contain stem cells which may be necessary for the induction and/or maintenance of a tolerant state through the establishment of chimaerisms.

In neonatal mice, thymocytes were reported to be inferior in conferring tolerance to allografts when compared with spleen cells or bone marrow cells (Billingham and Brent, 1959). In chickens, Ivanyi and Makings (1978) found that purified T lymphocytes failed to induce allogeneic tolerance whereas peripheral blood lymphocyctes and spleen cells were able to induce allogeneic tolerance. In these experiments both the host and donor chicken were juvenile from two different

populations differing by their minor histocompatibility antigens but not the major histocompatibility antigens. This seems to correspond with transplantation tolerance induced in metamorphosing (Chardonnens and Du Pasquier, 1973; Chardonnens, 1975; Du Pasquier and Chardonnes, 1975) or pre-metamorphic Xenopus laevis tadpoles (DiMarzo and Cohen, 1979) where tolerizing allografts were conditionally reported to be carrying identical major histocompatibility antigens but were probably disparate at the minor histocompatibility loci (usually at one locus; difference by two loci usually required the use of large grafts) (DiMarzo and Cohen, 1979). In Rana, on the other hand, it was possible to induce xenogeneic as well as allogeneic tolerance using skin cell homogenates from adult Rana temporaria injected into larval Rana temporaria or Rana arialis. These hosts were grafted with skin from the donor eight months later and were found to be tolerant (Vyazov and Sorokina, 1961, 1962). These results may simply reflect the difference in alloreactivity of the two species; Xenopus laevis on the one hand and the phylogenetically more advanced Rana temporaria or Rana arialis on the other. Or they may reflect the significance of the form and type of the antigen used. In higher vertebrates there is some experimental evidence that soluble alloantigens are potent inducers of both tolerance (Brent et al., 1973; Hilgert et al., 1974; Rao and Bonavida, 1976; Fevrier et al., 1978; Hasek et al., 1979) and positive anamnesis (Billingham et al., 1956).

In Xenopus laevis transplantation tolerance and/or anamnestic graft rejection may not be inducible by the infusion of adult blood leucocytes into tadpoles hosts and may require the presence of other tissues. Du Pasquier and his colleagues (1979) produced antibodies to allo-antigens in Xenopus laevis which required immunization with a combination of skin

graft application followed by several injections of leucocytes and RBC from the donor. Some of these animals required a second course of immunization, and even then the response was relatively weak. In mice also, the injection of allogeneic lymphocytes induced a longer lasting positive memory response for the immune elimination of allogeneic cells than for the rejection of skin grafts (Bainbridge, 1983).

Although skin grafts from adult Xenopus laevis can induce transplantation tolerance when applied onto metamorphosing individuals (Chardonnens and Du Pasquier, 1973), their blood leucocytes or thymocytes or splenocytes were ineffective in either preventing the development of such tolerance or in immunizing such hosts (Du Pasquier and Bernard, 1980). This is in spite of observing such cells persisting in the hosts' thymus and spleens. Immunologically competent allogeneic leucocytes are known to call forth a different type of host response from that elicited by allografts. It is also believed that transplantation tolerance is related to antigen persistence (chimaerism). The proportion of stem cells in an inoculum may therefore be an important factor.

- C H A P T E R S I X -

- C H A P T E R S I X -

ONTOGENETIC STUDIES ON IMMUNOLOGICAL MEMORY OF XENOPUS LAEVIS TO HUMAN GAMMA GLOBULIN INJECTED IN SALINE

6.1 INTRODUCTION

In the previous chapters it was demonstrated that both allogeneic blood leucocytes and xenogeneic erythrocytes were unsucessful in conferring a state of tolerance in Xenopus laevis. Both antigens were presented in cellular (particulate) form. It is thought however, that soluble forms of antigens may be more tolerogenic (Solomon, 1971). It is known that whether the antigen is presented before or after being processed by macrophages is an important factor in gearing the resulting memory towards tolerance or positive anamnesis, respectively (Unanue, 1972; Cowing et al., 1974; Oppenheim and Seeger, 1976; Oehler et al., 1978; Fritsh, 1981; Lukic, 1981). In mice, Auerbach (1975) was able to induce tolerance to sheep erythrocytes by injecting the recipients with a soluble lysate of the antigen (sheep erythrocytes); the intact cells were immunogenic. In the experiments described in the present chapter, human gamma globulin (HGG) was used as a representative soluble antigen.

Tolerance to HGG was induced in mice (Golub and Weigle, 1967), chicken (Grebenan and Thorbecke, 1978) and rabbits (Hunneyball and Stanworth, 1979) by injecting them with large doses of the antigen, solubilized in saline without the presence of adjuvant and aggregate free.

In Xenopus laevis the administration of HGG solubilized in saline did not provoke a good immune response unless given with Freund's Complete Adjuvant (Manning and Turner, 1972; Turner et al., 1974). Antigen localization in the spleen was also improved by the presence of the adjuvant with the immunogen (Collie, 1974; Collie and Turner, 1975). Adjuvants are thought to promote macrophage handling and processing of the antigen whereas a large dose of soluble and adjuvant free antigen is thought to reach the appropriate cells unprocessed, leading to a state of specific unresponsiveness (tolerance). In the experiments described in this chapter, an attempt was made to investigate whether it is possible to induce tolerance in Xenopus laevis tadpoles by injecting them with a relatively large dose of the antigen (HGG) dissolved in saline. The dose employed was forty times the standard dose usually used to immunize these animals (see Collie and Turner, 1975; Secombes and Manning, 1980). A similar attempt was also made using adult Xenopus laevis toads.

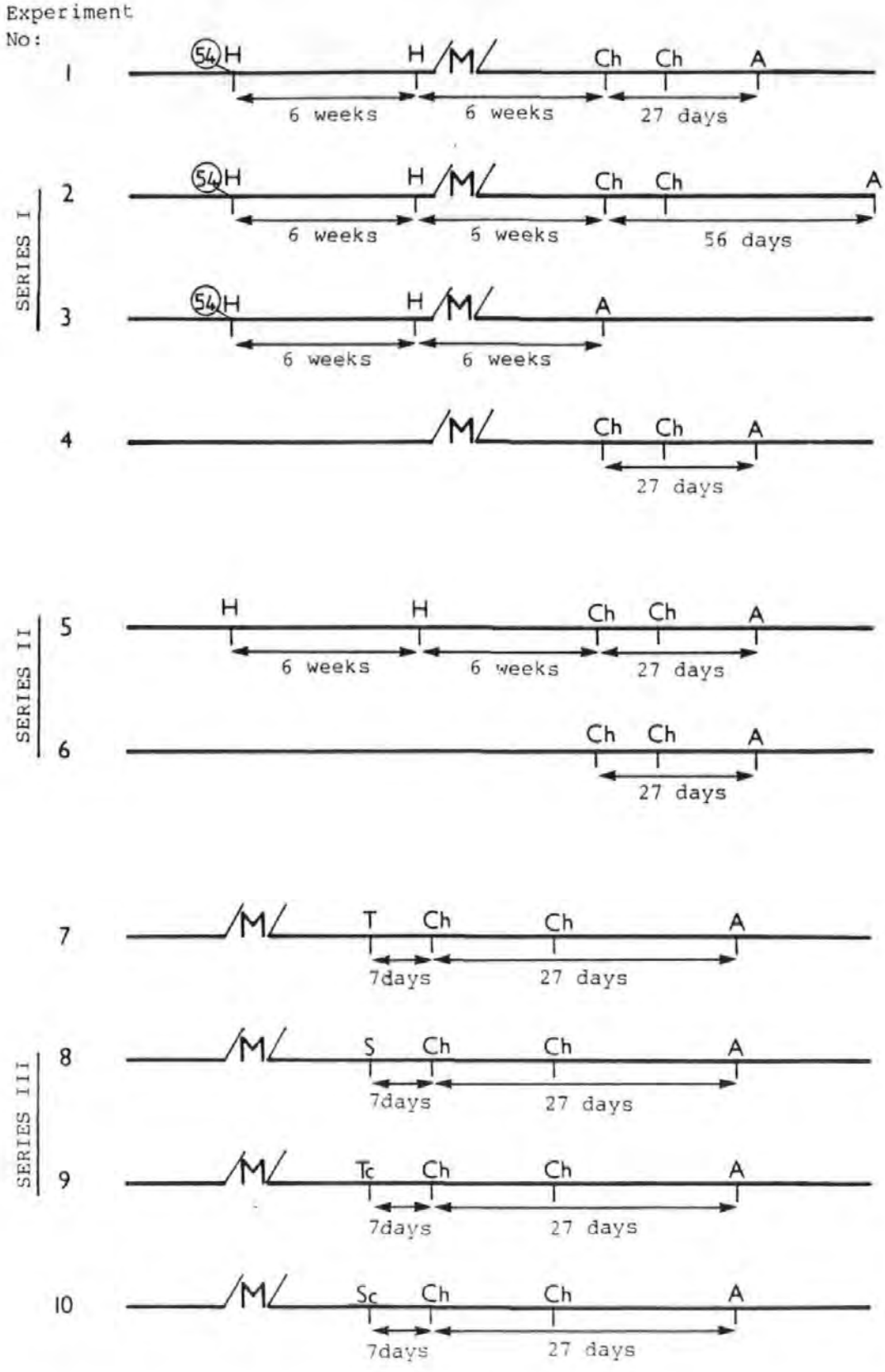
6.2 MATERIALS AND METHODS:

6.2.1 Experimental design:-

The protocol of these experiments is shown in Figure 14. The animals used in Series I and III described below were from the G line of Xenopus laevis which are histocompatible at the MHC, thus minimizing histocompatibility reactions in experiments involving the transfer of cells. The animals used in Series II were stock animals from our colony (see Chapter 2).

Figure 14 Experimental protocol for studying the effect of pre-treatment with human gamma globulin (HGG) in saline or cells from animals tolerant to HGG on antigen trapping in the spleen, antigen persistence in the serum and haemagglutinating antibody production in Xenopus laevis. Series I: toadlets treated with HGG in saline during larval life. Series II: adult toads given HGG in saline as toads. Series III: adoptive transfer of cells from tolerance animals. HGG in saline was given either at a high dose (1mg. g body weight^{-1}) or at a standard dose (0.025mg. g body weight^{-1}).

Key:
- A — Point of assay.
- H — Human gamma globulin (HGG) injection in saline :- 0.025mg. or 1mg. g body weight^{-1}.
- Ch — Challenge injection of HGG given with Freunds Complete Adjuvant (FCA) :- 0.025mg. g body weight^{-1}.
- 54 — Stage 54 of larval development (Nieuwkoop & Faber 1967).
- /M/ — Metamorphosis
- T,S — Stage of injecting thymocytes or splenocytes (respectively) from tolerant animals 5 x 10^5 cells.animal^{-1}.
- TC,SC — Stage of injecting thymocytes or splenocytes (respectively) from normal non-tolerant animals 5 x 10^5 cells.animal^{-1}.


Experiment No:
SERIES I
SERIES II
SERIES III
1
2
3
4
5
6
7
8
9
10
54
H
M
Ch
A
T
S
Tc
Sc
6 weeks
27 days
56 days
7days

SERIES I:

Twenty three tadpoles at Stage 54 of their development (Nieuwkoop and Faber, 1967) were injected intraperitoneally with a dose of 1mg.g body weight^{-1} of human gamma globulin (HGG) dissolved in 0.85% saline (this is termed a high dose). Six weeks later, this high dose was repeated and six weeks after that, during which time the tadpoles had metamorphosed into toadlets, four animals were sacrificed to provide a sample for monitoring the persistence of antigen. These animals were tested for the presence of trapped antigen in their spleens using the direct immunofluorescence technique described below. Their sera were also sampled for the presence of persistent antigen, using an Ouchterlony double diffusion precipitation test in agar gel (see below).

The remaining toadlets were challenged using two injections, twelve days apart, of 0.025mg.g body weight^{-1} HGG in adjuvant. This is known, from experience in our laboratory, to be a strongly immunogenic dose for normal animals. The antigen was prepared for injection by adding one volume of a 1mg.cm^{-3} solution of HGG in 0.85% saline to an equal volume of Freund's Complete Adjuvant and mixing thoroughly by vigorous shaking. Each animal received 5µl.g body weight^{-1} delivered via the dorsal lymph sac.

Four and 15 animals were sacrificed at day 27 and day 56 respectively, after the day of the first challenge. Their spleens were tested for the presence of trapped antigen, using immunofluorescence and their sera titrated for anti-HGG antibody levels employing the passive haemagglutination technique described below. A further two animals, which had been treated by high dose injections as tadpoles, were reserved for the experiments described in Series III.

In parallel with the experiments employing the high dose of antigen, a further eight animals were subjected to the same schedule of treatment but were given the standard immunogenic dose of 0.025mg. HGG of body weight^{-1} (delivered in saline) in place of the high dose. Five and three animals were sacrificed at day 27 and day 56 respectively after the day of the first of the two challenge injections of HGG in adjuvant, their spleens and sera were tested alongside those from animals which received the high dose. Eight animals received the two challenge injections of 0.025mg. HGG g body weight^{-1} in adjuvant alone, i.e. without any prior injections earlier in their development. These were killed at day 27 and their spleens and sera tested as above. A further 11 untreated toadlets were tested in the immunofluorescence, passive haemagglutination and double diffusion precipitation assays to provide normal controls for these tests.

SERIES II:

In this series, the experiments followed a similar schedule to that employed in Series I but instead of commencing at the larval stage of development, the animals were already adult toads at the beginning of the experiment. Five toads received the high doses of antigen, while seven toads received the standard immunogenic doses. All toads were killed at day 27 after challenge.

SERIES III:

These experiments were designed to see whether any transfer of tolerance could be detected when cells from tolerant animals were transferred to normal recipients. The animals used in these experiments were MHC histocompatible siblings from the G-line of Xenopus laevis.

Thymocyte or splenocyte suspensions were prepared, taken from either untreated toadlets or from toadlets which had received high doses of HGG as tadpoles (potentially tolerant animals). These cells were injected into the dorsal lymph sac of the recipient.

To prepare the cell suspensions, the donors were sacrificed, their thymuses and spleens were excised and placed in a volume of diluted culture medium in a watch glass on ice. The organs were then teased apart with a pair of sterile fine, solid needles and transferred into a sterile manual glass tissue homogenizer where they were carefully and gently ground (to avoid damaging too many cells). The suspension was allowed to stand in a thin tube, placed in ice for a few minutes until the large debris sedimented on the bottom of the tube before transferring the supernatant (containing the cells) to a centrifuge tube. This was centrifuged three times, each time replacing the supernatant with fresh cold medium. The number of cells in the resulting suspensions were estimated using a haemocytometer. Their viability was determined using the nigrosine dye exclusion test in which 0.1 cm^3 of the cell suspension was mixed with 0.1 cm^3 of 0.2% nigrosine solution and incubated at room temperature for five minutes. Viable cells (i.e. those which excluded the dye) were estimated to average about 95% of the cells in the suspensions.

The recipients were arranged in eight groups of two animals per group, the two animals of a pair receiving cells from the same donor organ. Each toadlet received 5×10^5 donor cells injected via the dorsal lymph sac. (In the case of one pair which had received splenocytes from a tolerant donor, the spleens were unfortunately lost during preparation but the sera were collected and tested). Two donors which had been

injected previously with high doses of HGG followed by challenge (see Series I) were used in these experiments. Each spleen and thymus provided cells for two recipient toadlets (8 recipients in total). Eight control recipients received similar cells from two untreated donors. The sera from the donors was assayed for the presence of anti-HGG antibodies using the passive haemagglutination test. No antibody was detected suggesting that high zone tolerance had been achieved.

A week after receiving donor cells, the recipients were injected with the first of two challenge doses of 0.025g body weight^{-1} HGG in adjuvant. The second similar injection was given 12 days later and the animals were killed 15 days after the second injection for testing by immunofluorescence and passive haemagglutination assays.

6.2.2 Direct immunofluorescence tests:-

The spleens were excised from animals which had been killed in MS222 (2g 400cm^{-3} MS222 in water). They were then embedded in a portion of liver which had been removed from the same animal and placed on a moist disc of filter paper on top of a microtome chuck. The chuck, bearing the tissues, was mounted on a J-shaped metal holder and carefully lowered into liquid nitrogen. The tissues were kept above the surface of the fluid because direct immersion in liquid nitrogen results in cracks appearing in the tissues making them difficult to cut. When the liquid nitrogen had stopped boiling, the chuck was removed, mounted onto the cryostat microtome and left for the tissues to equilibrate to the cryostat temperature (-17^{o}C). Sections were then cut and mounted onto 25mm x 76mm glass microscope slides, which had previously been thoroughly cleaned in acid alcohol. The sections were then air dried in front of a

fan for half an hour and ringed as an aid to locating the section under the fluorescence microscope, the ring being drawn with a diamond pencil on the under-side of the slide around each section.

A stock solution of fluorescein labelled anti-human immunoglobulin (anti-HGG) antiserum produced in sheep was made up according to the manufacturer's directions (Wellcome, Beckenham, Kent). This was further diluted just before use to provide a 1:15 (v/v) solution in phosphate buffered saline pH 7.2. A drop of this antiserum was placed on each section and the slides were then incubated in a moist chamber at room temperature for 30 minutes. After incubation, the slides were lightly blotted, placed in a slide holder and immersed suspended in a bath of phosphate buffered saline pH 7.2 which was stirred on a magnetic stirrer, the slides were then washed for one hour changing the buffer once after the first half hour. After washing the slides were taken out, excess buffer was blotted off and the sections were mounted in a glycerol/ phosphate buffered medium pH 7.2. This fluid was prepared by mixing one volume of phosphate buffered saline pH 7.2 with 9 volumes of glycerol, adjusting the pH to 7.2 with 0.1M solution of sodium hydroxide when necessary.

The mounted slides were placed in dark moist chambers until examined with a fluorescence microscope (Vickers) on the same day. Photographs of the positive sections were taken on 400 ASA black and white films of slightly high contrast (Ilford, HP5 and HP4).

6.2.3 Passive haemagglutination assay:-

This assay was basically a haemagglutination assay, except that the sheep erythrocytes used were coated with either the test antigen,

an unrelated control antigen or saline (uncoated).

The sera were obtained and prepared, as described in Chapter 3, for the haemagglutination assay, but in addition to heat inactivation they were also absorbed with sheep erythrocytes. Each volume of serum was added and mixed with an equal volume of washed and packed erythrocytes. After one hour incubation at room temperature, the sera were separated from the erythrocytes by centrifugation at 2200g for 5 minutes. These sera were stored at $4^{o}C$ until the time of the test on the same day, or stored frozen at $-20^{o}C$ for longer periods. Care was taken not to commence the absorbtion process before the sera had cooled down to room temperature to prevent erythrocytes lysis by the hot sera.

Another difference between passive and direct haemagglutination assays is that the solution used to serially dilute the test sera (the diluent) is saline or phosphate buffered saline in the direct assay and a 1:100 (v/v) dilution of normal heat inactivated and absorbed Xenopus laevis serum in saline in the passive haemagglutination assay. This acts as a stablilizer to prevent spontaneous agglutination of the tanned erythrocytes.

The test antigen used in this study was human gamma globulin (HGG) whereas the control antigen was ovalbumin (used to indicate the specificity of the anti-HGG positive sera reactions). The erythrocytes coated with saline (not coated with antigen) were used as a control for the effect of the coating process itself and to check for spontaneous agglutination.

The erythrocytes were washed three time with saline, packed and resuspended in phosphate buffered saline pH 7.2 to a concentration of 2.5% (in volumes). To each volume of erythrocyte suspension an equal volume of 0.005% solution of tannic acid in saline was added and incubated for 5 minutes at 37°C. After incubation the cells were washed twice with phosphate buffered saline pH 7.2 and resuspended in this buffer to the original concentration (2.5%). These tanned cells were coated with the antigens by mixing each volume of tanned cell suspension with four volumes of phosphate buffered saline pH 6.4 and one volume of either saline or a $1mg.cm^{-3}$ solution of the antigen (either HGG or ovalbumin). This mixture was incubated for 10 minutes at room temperature after which the coated cells were washed once with diluent (1% solution of normal Xenopus laevis serum) and resuspended to a concentration of 0.25% in this solution. This final suspension was then used in the tests following the same procedure as for the haemagglutination assay: 50µl/well were added to the serially diluted sera in diluent. The plates were sealed with scotch tape, vigorously shaken on a horizontal surface and left on the bench for 3 hours before storing at 4°C over night. Next morning the plates were read, the last well to show distinct agglutination being taken as the end point of the titration.

6.2.4 Agar double diffusion test:-

The agar gel plates were prepared as follows:-
0.206g of barbitone acetate buffer (Oxoid, London) were dissolved in 6.25 cm^3 of warm distilled water. The solution was then cooled and a further 6.25 cm^3 of distilled water were added. An agar tablet (I.D. Agar, Oxoid, London) was added to the solution which was then made up

to 50 cm^3 by adding more distilled water. It was boiled with stirring until the agar was completely dissolved. The hot viscous solution was then placed in a 56°C oven to allow for the exclusion of any air bubbles. Half an hour later the agar was poured into 15 x 100mm plastic petri dishes to a thickness of 2-3mm on a horizontal surface and allowed to solidify at room temperature.

A plastic template was used to cut a pattern of four holes in the agar with a metal borer. One central hole was surrounded by three equally spaced holes. Since in the present studies this test was used for the detection of persisting human gamma globulin in the sera of immunized Xenopus laevis toads and toadlets, the central hole was filled with sheep anti-human whole serum antiserum which includes an anti-HGG component and an anti-HSA component (Wellcome, Beckenham, Kent). One of the surrounding holes was filled with the test serum while the other two were filled with $1mg.cm^{-3}$ solution of human gamma globulin in one well and $1mg.cm^{-3}$ solution of human serum albumin (HSA) in the other well (as positive controls).

The preparations were placed in a moist chamber at 4°C and incubated for seven days after which they were examined for the development of white precipitation lines, indicating positive reactions. A line of identity was formed with the known HGG well and a line of non-identity with the albumin well when the line from the test well was positive.

6.3 RESULTS:

6.3.1 Responses of untreated toads and toadlets to challenge with HGG in adjuvant:

Series No. (Group No)	Dose of HGG given before challenge (mg.g body weight^{-1})	Day of test (day after first challenge injection)	HGG in splenic white pulp (immunofluorescence)	Serum antibody titres ($-\log_2$)	HGG in the serum (- indicates that none was found)
I (1)	none	NC	-,-,-,-,-,-,-	0,0,0,0,0	-,-
I (2)	none	27	2+,2+,2+,2+,2+,2+, 2+,1+	0,0,0,4,4,4,5, 6	N.D.
I (3)	1	27	-,-,-,-	0,0,0,0	N.D.
I (4)	1	56	-,-,-,-,-,-,-,-,-, -,-,-,-	0,0,0,0,0,0,0,0, 0,0,0,0,3,8,9	N.D.
I (5)	1	NC	-,-,-,-	N.D.	-,-,-,-
I (6)	0.025	27	-,-,-,-	8,9,9,11	N.D.
I (7)	0.025	56	-,-,-	3,7,11	N.D.

Table 12 Antigen retention in both splenic white pulp and serum, and serum antibodies in Xenopus laevis toadlets given human gamma globulin (HGG) in saline in standard (0.025mg. g body weight^{-1}) or high (1mg. g body weight^{-1}) doses as tadpoles. The animals were challenged with two injections of HGG in Freund's Complete Adjuvant (FCA) (0.025mg. HGG g body weight^{-1}) and tested either 27 days or 56 days after the first challenge. Each figure represents one reading.

Key: NC - Not challenged.

\- - No fluorescence (1+,2+,3+) arbitrary score indicating weak (1+) to strong (bright) (3+) fluorescence.

ND - Not done.

These results are from Experiments 1-4 in Figure 14.

Series No (Group No)	Dose of HGG given before challenge (mg.g body weight^{-1})	Day of Test	HGG in splenic white pulp (immunofluorescence)	Serum antibody titres ($-\log_2$)
(1)	none	NC	-,-,-,-,-,-	0,0,0,0,0,0,
II (2)	none	27	2+,2+,3+,3+	8,9,10,10
II (3)	1	27	-,-,-,-,1+	0,0,0,0,0
II (4)	0.025	27	-,-,-,1+,1+,1+,1+	0,0,5,6,7,8,8

Table 13 Antigen retention in splenic white pulp and serum antibodies in Xenopus laevis adult toads given human gamma globulin (HGG) in saline in standard (0.025mg.g body weight^{-1}) or high (1mg.g body weight^{-1}) doses as toads 6 weeks and 12 weeks before challenge. The animals were challenged with two injections of HGG in Freund's Complete Adjuvant (FCA) (0.025mg. HGG g body weight^{-1}) and tested 27 days after the first challenge. Each figure represents one reading. These results are from experiments 5 and 6 in Figure 14.

NC Not challenged

\- No fluorescence (1+,2+,3+) arbitrary score indicating weak (1+) to strong (bright) (3+) positive fluorescence.

Figure 15 Serum antibody titres (mean $\pm$ standard error) in Xenopus laevis given human gamma globulin (HGG) in saline in standard (0.025mg. g body weight^{-1}) or high (1mg. g body weight^{-1}) doses. Primed toadlets received their primary injections as tadpoles and were later challenged as toadlets. Primed toads received their primary injections as toads and were later challenged. The challenge injections consisted of HGG (0.025mg. g body weight^{-1}) injected with Freund's Complete Adjuvant (FCA). The animals were sacrificed at 27 days after the first challenge injection, unless otherwise shown in the figure. Control animals were either not injected at any stage or challenged only. These results are from Experiments 1,3-6 in Figure 14.

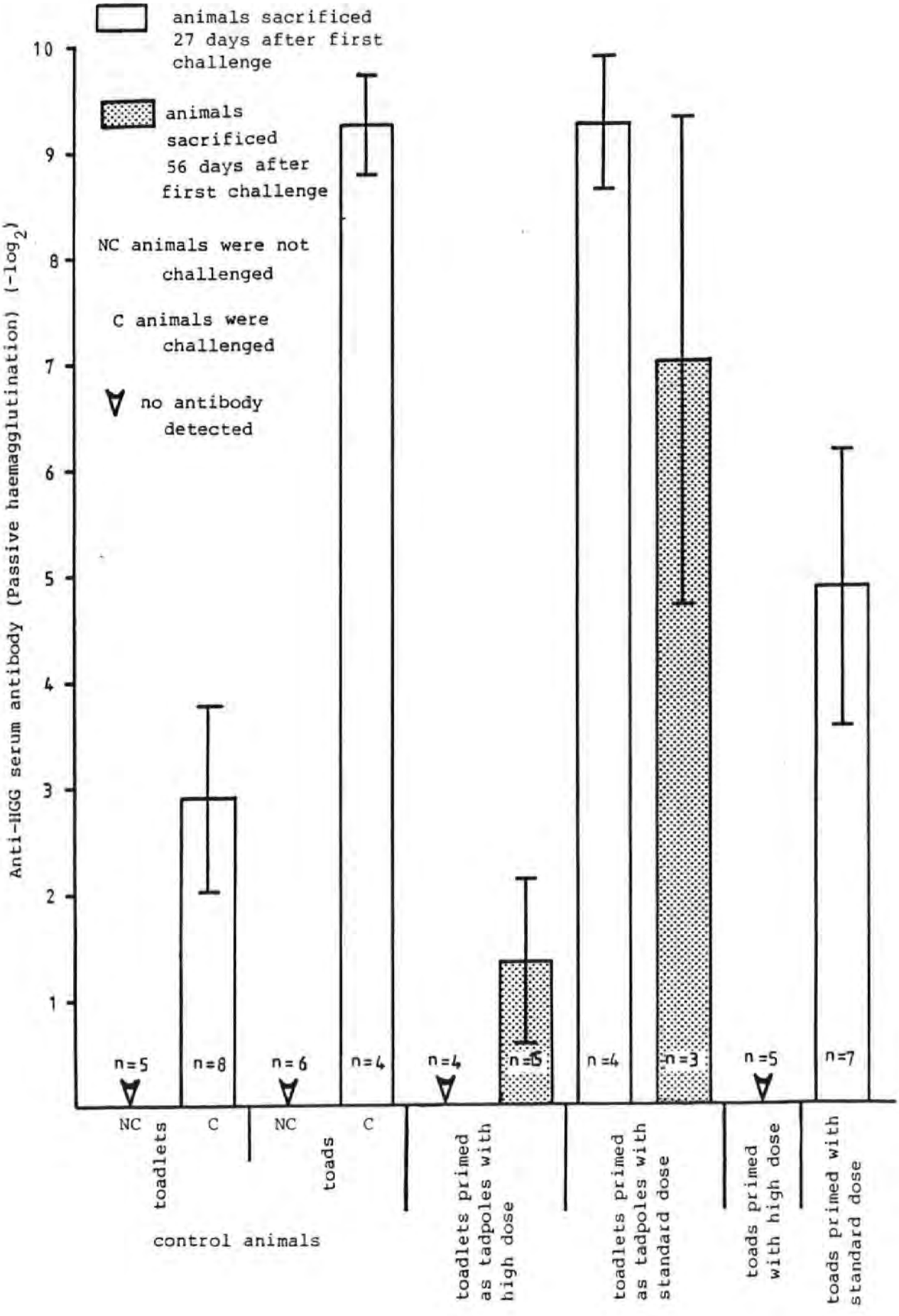
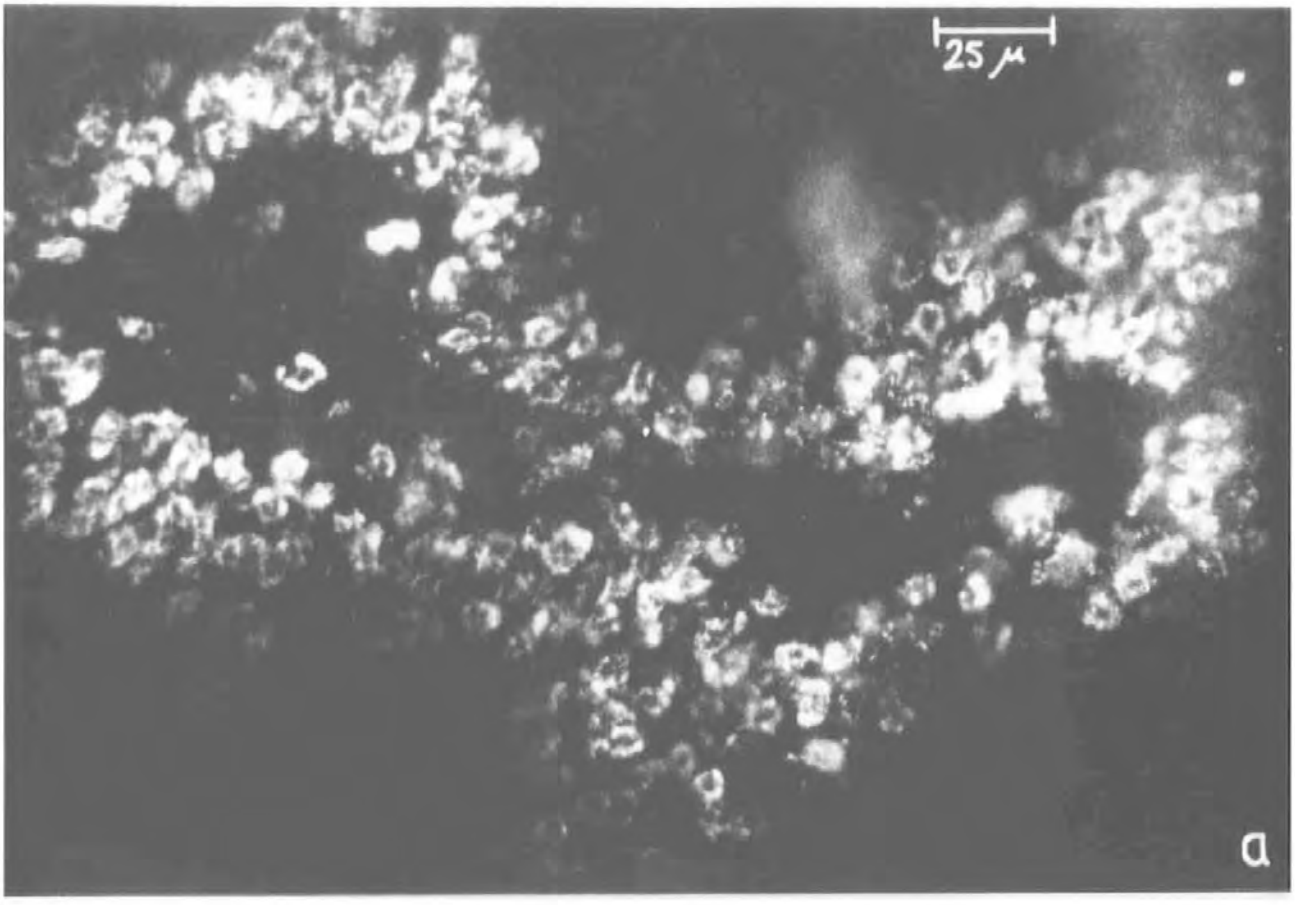
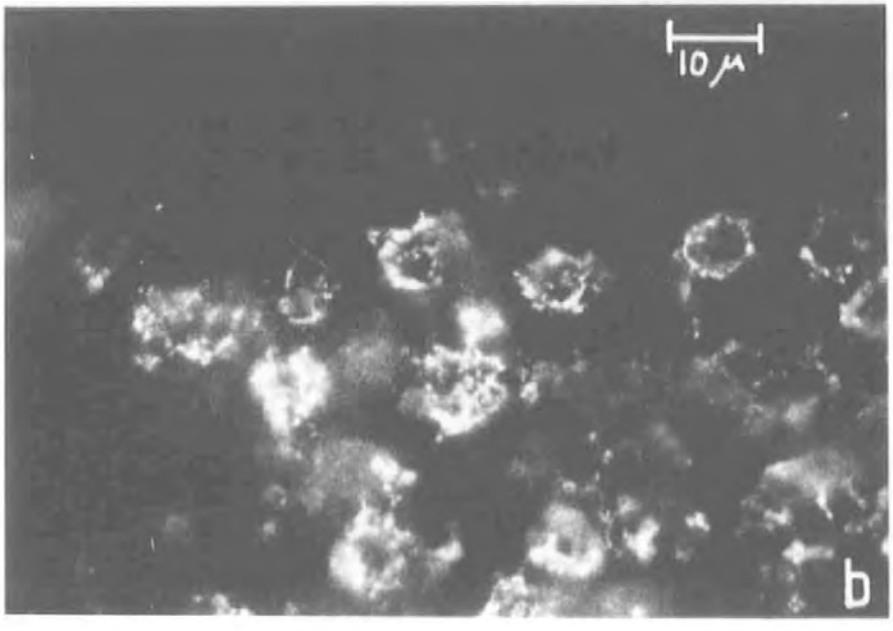

animals sacrificed 27 days after first challenge
animals sacrificed 56 days after first challenge
NC animals were not challenged
C animals were challenged
no antibody detected
Anti-HGG serum antibody (Passive haemagglutination) (-log2)
10
9
8
7
6
5
4
3
2
1
n=5
n=8
n=6
n=4
n=4
n=5
n=4
n=3
n=5
n=7
NC
C
NC
C
toadlets
toads
control animals
toadlets primed as tadpoles with high dose
toadlets primed as tadpoles with standard dose
toads primed with high dose
toads primed with standard dose

Figure 16 Immunofluorescence picture in the spleen of a Xenopus laevis toad injected with 0.025mg. g body weight^{-1} HGG with adjuvant at day 27 and day 15 before sacrifice. Fluorescence indicates the site of antigen trapping within the white pulp of the spleen.

(a) Antigen localization in the outer zone of the white pulp.

(b) View at higher magnification to show the distribution of antigen on the surface of cells.


25 μ
a


10 μ
b

A. Antigen localization in the spleen:

Challenged, but otherwise untreated, toadlets and toads (Group 2 in Table 12 and Group 2 in Table 13) were found to trap antigen (HGG) in the white pulp of their spleen when tested using direct immunofluorescence at 27 days after the first challenge injection. Fluorescence was observed as intercellular or dendritic depositions in the form of a ring in the outer zone of the white pulp but within its boundary layer, as has been described previously by Horton and Manning (1974), Collie and Turner (1975) and Secombes and Manning (1980) (see Figure 16). The fluorescence was brighter in the adult toads than in toadlets. It was never observed in the spleens of normal, untreated animals.

B. Serum anti-HGG antibodies:

The difference in the intensity of splenic antigen trapping between challenged, but otherwise untreated, toads and toadlets was matched by similar differences in their serum anti-HGG antibody levels, the titres obtained for toads and for toadlets being 1:512 ($-\log_2=9$) and 1:8 ($-\log_2=3$) respectively (see Figure 15). No antigen or antibody was detected in the sera of normal (untreated and unchallenged) animals and none of the sera agglutinated the uncoated SRBC or the SRBC coated with ovalbumin (OVA).

6.3.2. Responses to HGG of challenged toadlets previously exposed to HGG as tadpoles:

The results of these experiments are shown in Table 12 (experiments 3-7) and in Figure 15.

A. Antigen localization in the spleen:

No fluorescence, such as might indicate the presence of trapped antigen, was detected in the spleens of toadlets which had previously received HGG as tadpoles, irrespective of whether this antigen had been delivered at high dose (1mg.g body weight^{-1}) or standard dose (0.025mg.g body weight^{-1}) levels. This was the case whether the animals were tested at 27 days or at 56 days after the first challenge injection (see Table 12).

B. Serum anti-HGG antibodies:

Toadlets which had been treated with the standard immunogenic dose of HGG as tadpoles and tested 27 days after the first challenge injection showed anti-HGG antibody activity in their serum. The mean titre was 1:512 ($-\log_2 = 9$) (see Table 12 and Figure 15). This is higher than the titres produced by challenged, but otherwise untreated, toadlets and is closer to the adult levels of antibody production. In one animal of this group, no spleen could be found and this animal showed no antibody production. Antibody was still present in the serum of animals sampled 56 days after the first challenge injection (Table 12 and Figure 15).

In contrast, no anti-HGG antibodies were detected in the sera of toadlets treated with the high doses of HGG as tadpoles and killed at day 27, after the first challenge injection. Of those killed at day 56 following challenge, again twelve of the fifteen animals showed no antibody production; the remaining three animals gave antibody titres of 1:8 ($-\log_2 = 3$), 1:256 ($-\log_2 = 8$) and 1:512 ($-\log_2 = 9$).

The use of Ouchterlony double diffusion tests failed to detect any persistent HGG antigen in the sera of four toadlets, which had been treated with high doses of antigen as tadpoles and which were killed without challenge at the time when toadlets in the main experimental series, received their challenge injection.

6.3.3 Responses to HGG of challenged toads following prior exposure to HGG:-

The toads had been treated with high or standard doses of HGG in saline following a schedule similar to that used in Section B but applied at the adult stage of development rather than to tadpoles. They were killed 27 days after the first challenge injection. The results are shown in Table 13and Figure 15.

A. Antigen localization in the spleen:

Immunofluorescence tests detected antigen in the spleen of one out of five toads treated with the high dose of HGG before challenge, and in four out of seven toads given standard doses before challenge. The intensity of fluorescence in the positive antigen localizations was not very strong (see Figure 17).

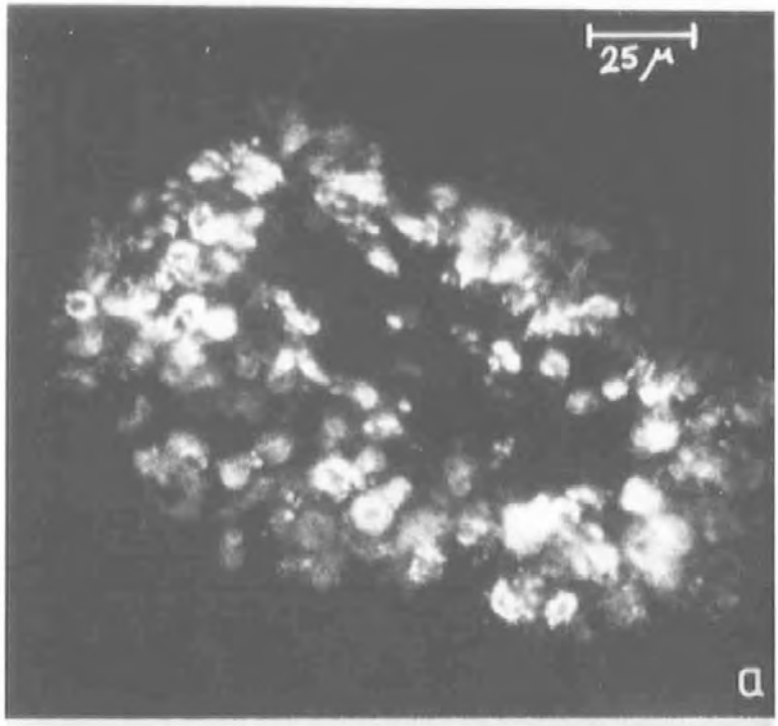
B. Serum anti-HGG antibodies:

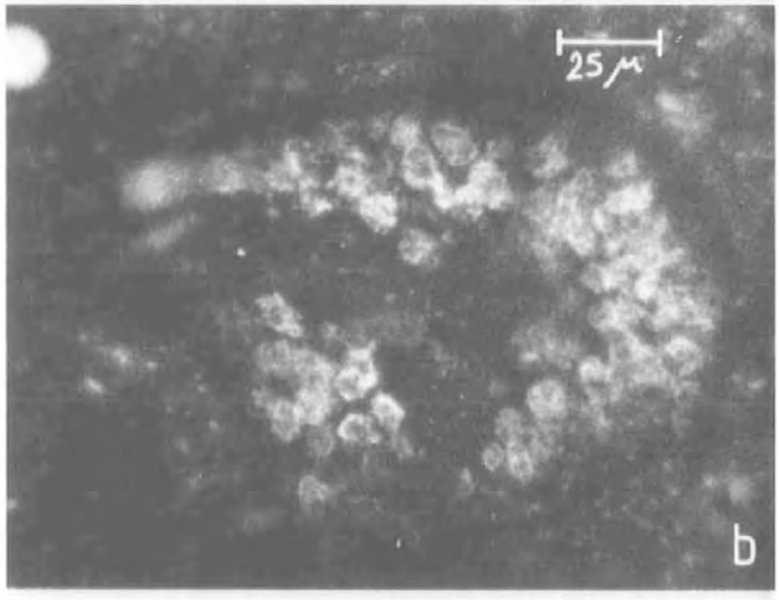
No antibodies were detected in the sera of toads treated with high doses of HGG before challenge, while those treated with standard immunogenic doses before challenge showed a mean titre of 1:32 ($-\log_2 = 5$) with two animals failing to produce any detectable antibody. In general, the antibody response of both groups was either impaired or reduced,

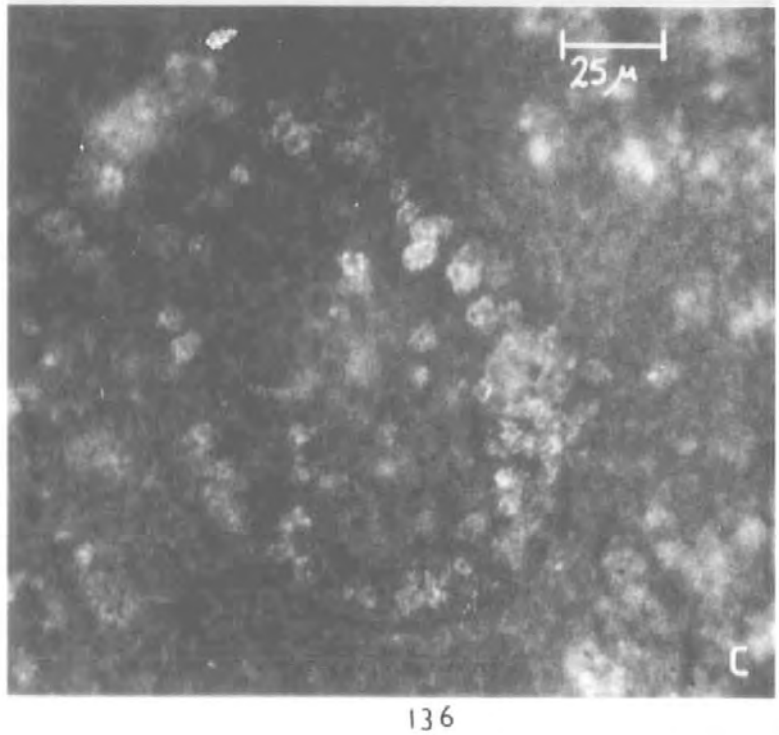
Figure 17 Immunofluorescence picture showing different intensities of fluorescence in the white pulp of Xenopus laevis toads.

(a) Spleen from a toad injected with 0.025mg. g body weight^{-1} HGG in adjuvant at day 27 and day 15 before sacrifice (rated 3+ in Table 13).

(b) and (c) Spleens from toads previously primed with standard doses (0.025mg. g body weight^{-1}), then challenged with 0.025g body weight^{-1} HGG in adjuvant at day 27 and day 15 before sacrifice (rated 1+ in Table 13).


25 μ
a


25 μ
b


25 μ
c

compared with the challenged, but otherwise untreated, toads described in Section 6.3.1 above.

The use of Ouchterlony double diffusion tests in toads sampled six weeks after receiving high doses of HGG (but not challenged) showed that HGG was still present in the serum (three toads). None was detected in two toads which received standard doses of antigen.

6.3.4 Attempts to Transfer the acquired unresponsiveness to HGG:-

The results of this experiment are shown in Table 14.

All recipients were able to trap antigen (HGG) in their spleen, irrespective of whether they had been treated with spleen cells or with thymus cells and whether or not the cells had been obtained from an unresponsive high dose treated donor. The presence of the antigen was shown by medium (2+) to very bright (3+) fluorescent rings in the white pulp of the spleen (see Figure 18).

Despite their ability to localize antigens in the spleen, antibody production in the recipients was for the most part lacking (see Table 14). The two animals which did show the presence of anti-HGG antibody in their serum had in fact been treated with spleen cells from a high dose treated, tolerant donor. The reason for the suppression of antibody production is not known but it should be noted that this occurred in toadlets given cells from normal animals as well as in those receiving cells from tolerant donors. This, together with the positive splenic antigen localization, lends little support for any transfer of tolerance in this investigation, although it is clear that further experiments are

Series No	Type and Source of cells injected	HGG in splenic white pulp (immunofluorescence) *	Serum antibody titres ($-\log_2$)
III	TC	2+,3+,3+,3+	0,0,0,0
III	SC	2+,3+	0,0,0,0
III	T	1+,2+,2+,2+	0,0,0,0
III	S	1+,1+,2+,2+	0,0,3,7

Table 14 Antigen retention in splenic white pulp and serum antibody titres in Xenopus laevis toadlets given thymocytes or splenocytes either from donors tolerant to human gamma globulin (HGG) (T or S respectively). The animals were challenged one week after receiving the cells with two injections of HGG in Freund's Complete Adjuvant (FCA) (0.025mg HGG g body weight^{-1}) and tested 27 days after the first challenge.

*: (-) no fluorescence. (1+,2+,3+) arbitrary score indicating weak (1+) to strong (bright) (3+) positive fluorescence.

These results are from Experiments 7-10 in Figure 14.

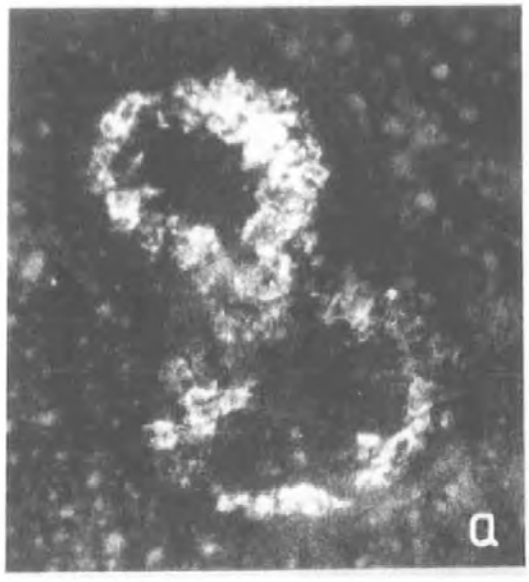
Figure 18 Immunofluorescence picture in the spleens of Xenopus laevis toadlets injected with 0.025mg. g body weight^{-1} HGG with adjuvant at day 27 and day 15 before sacrifice.

(a) Toadlet given splenocytes from a normal toadlet donor one week prior to challenge.

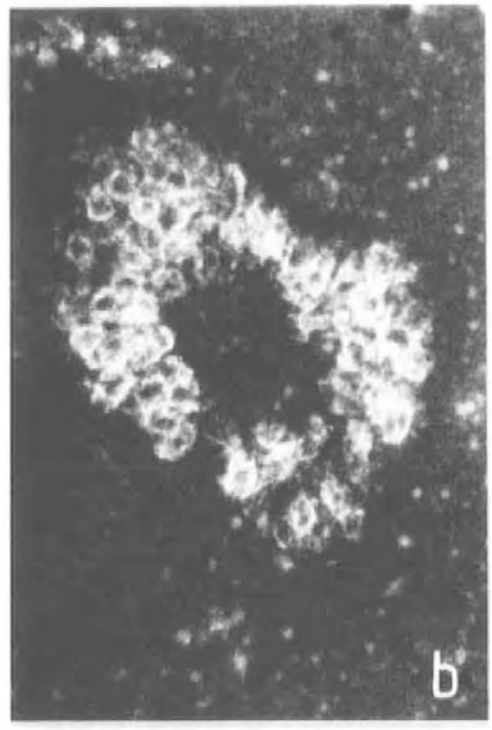
(b) Toadlet given splenocytes from a toadlet donor tolerant to HGG one week prior to challenge.

(c) Toadlet given thymocytes from a normal toadlet donor one week prior to challenge.

(d) Toadlet given thymocytes from a donor toadlet tolerant to HGG one week prior to challenge.



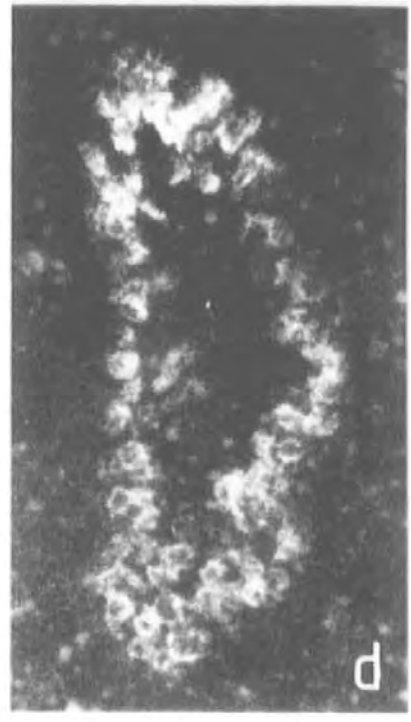
25 μ



25 μ

50 μ

25 μ



required.

6.4 DISCUSSION:

The results described in this chapter suggest that the injection of high doses of HGG dissolved in saline can be tolerogenic when administered either to Xenopus laevis tadpoles (commencing at Stage 54 of larval development) or to adult toads. Thus, when the animals were challenged six weeks after the last primary injection, they were found to be deficient both in their antibody production and in their ability to trap antigen in the spleen. This latter reaction, which probably involves the localization of immune complexes, is believed to be antibody dependent and to require the presence of immunocompetent T-cells (Horton and Manning, 1974; M.S. Mughal, 1984). The findings are not unequivocal since it is a general observation from our laboratory that toadlets can be more erratic than adult toads in the intensity of antigen trapping and in the levels of antibody which they produce. Nevertheless, the results show a difference between groups pre-treated with high doses of antigen and those pre-treated with standard doses, which suggest that some degree of high zone tolerance had been induced. Furthermore, these results have recently been confirmed by Mughal (1984).

In higher vertebrates tolerance to soluble antigens is thought to be governed by one or more of several mechanisms; clonal deletion of mature immunocompetent cells (Fidler, 1979; Grebenau et al., 1979; Parks and Weigle, 1980a, 1980b) clonal abortion of immature lymphocytes (Nossal and Pike, 1975; Stocker and Nossal, 1977), active suppression

(Parks et al., 1978; Grebenau and Thorbecke, 1979; Waters et al., 1979) and passive suppression by serum blocking factors (Wright et al., 1973, 1974, 1975, 1977; Auerbach, 1975; Morgan, 1978; Morgan et al., 1978, 1979; Fidler, 1979). The latter factors were further categorised into antigen-antibody complexes (Wright et al., 1973, 1974, 1975, 1977; Morgan, 1978; Morgan et al., 1978, 1979), antigen (Louise et al., 1973; Nossal, 1974) or other factors (Morgan, 1978; Morgan et al., 1979).

In an attempt to study tolerogenic mechanisms in Xenopus laevis, spleen or thymus cells from tolerant animals were transferred to normal toadlets. The adoptive transfer was unsuccessful, possibly indicating that suppressor cells were not involved. The results were difficult to interpret, however. Antigen trapping was not abrogated in any group, but the cell transfer depressed antibody production irrespective of the status of the donor animal. Whether this represents some form of antigenic competition (the donor lymphocytes might be seen as a foreign antigen, because although identical at the MHC, there is disparity with the G-line in the minor histocompatibility antigens), or whether the injection of allogeneic cells one week before the administration of HGG had some other effect on normal antibody production is unknown. Clearly further experiments are needed in order to interpret these results.

Other types of suppression, such as the presence of free antigen-antibody complexes in the serum may be involved. Such factors are thought somehow to block the activation of effector antibody producing cells in chicken (Morgan et al., 1978).

Persisting antigen in the circulation of tolerant animals may also govern their state of tolerance. This may operate in one of two possible

ways; either by blocking specific antigen recognition receptors (Borel and Aldo-Benson, 1974) or by binding to the antibody produced thus masking it from detection in passive haemagglutination tests. As the results show, antigen was not detected in the sera of animals rendered tolerant to HGG as tadpoles when tested in Ouchterlony plates. In toads however, persisting antigen could still be detected in the serum by Ouchterlony double diffusion tests, six weeks after high doses of HGG were injected. Whether this is an indication that tolerance induction in larvae and adults is mediated by different mechanisms is not clear. Presumably antigen injected on a "weight of antigen per body weight of animal" basis soon becomes diluted in the rapidly growing tadpoles and is less likely to be detectable later on.

Toadlets which had received the standard dose of HGG in saline as tadpoles showed elevated antibody titres which may indicate a state of positive memory (ρ 0.001 when group 6 is compared with group 2 in Table 12). It is of interest that the tadpoles were not rendered tolerant by standard doses of antigen, but only by high doses which were tolerogenic in adult animals also. In adults, on the other hand, pre-treatment with standard doses (delivered in saline) did not induce positive memory and may even have slightly suppressed the immune response.

The fact that toadlets which had received the standard dose of HGG in saline as tadpoles could, apparently, produce a positive memory response in the absence of any antigen trapping in the spleen may suggest that, unlike higher vertebrates (Klaus et al., 1980), antigen trapping in the spleen may not be necessary for the development of memory cells in Xenopus laevis. Antigen trapped on the surfaces of dendritic cells in the spleens of higher vertebrates is believed to

persist there for long periods of time (Mandel et al., 1980). This is thought to regulate and maintain antibody production to the antigen (Tew et al., 1980). Antigen trapping in Xenopus laevis is known to be efficient (Horton and Manning, 1980). It was observed to take place in the spleen and the kidney (Collie, 1976). Studies on the kinetics of this process showed that antigen could first be detected in the red pulp surrounding the white pulp of the spleen at half an hour post-immunization (Collie, 1974), the antigen (HGG) was injected in saline via the dorsal lymph sac. Thirty minutes later, another area of fluorescence (indicating an area of HGG localization) was detected in the splenic white pulp. The fluorescence in the red pulp disappeared completely after four days, leaving that in the white pulp in the form of a bright ring within the white pulp and close to the boundary layer. A closer look at this final picture revealed that fluorescence could be identified on the surfaces of large pyroninophilic cells with dendritic characteristics and also on small lymphocytes adjacent to them (Horton and Manning, 1974). More precise studies of these cells suggest that they act in transporting the antigen from the red pulp area to the white pulp area through the boundary layer (Baldwin and Cohen, 1981a, 1981b). The mechanisms of these processes and their function is not yet certain.

Collie (1974) and Collie and Turner (1975) have observed that, depending on the dose of HGG injected into Xenopus laevis toads, fluorescence in the spleen disappears at various times; eight weeks for a dose of 0.0025 mg/gm body weight and 12 weeks for 0.025 mg. At all doses however, the antigen was observed to disappear from the serum before it did from the spleen (Collie and Turner, 1975). Antigen trapping in the white pulp, unlike that in the red pulp, was found to be thymus dependent and does not occur after early thymectomy (Horton and

Manning, 1974; Collie, 1974). This may lead to the tentative suggestion that the tolerance observed in the present experiments involves thymus dependent populations of cells.

It is known that Xenopus laevis can produce antibody in the absence of splenic localization of antigen because antibody formation can still be elicited in splenectomized Xenopus laevis except when very low doses of antigen are involved (Turner, 1973; Collie and Turner, 1975).

The occurrence of antigen trapping in the absence of antibody (e.g. Table 14) is more surprising because it is believed that the antigen is localised in the form of antigen/antibody immune complexes. It is possible however, that only low levels of antibody are required for this immune complex formation.

Bufo marinus toads, tolerant to high doses of bovine serum albumin (BSA), could not eliminate a challenge dose of this antigen by immunological mechanisms but only metabolically (Marchalonis and Germain, 1971, 1980). The technique employed by these authors (the clearance of radioactively labelled antigen) is more sensitive than the precipitation tests used in the present study. Such techniques used with Xenopus laevis might reveal whether the tolerance to HGG induced in adult toads (see Table 13) is merely due to the presence of free antigen in the serum masking any antibody which may be produced. Bufo marinus produces both IgM and low molecular weight antibodies. The ability to produce a memory response, whether positive memory or tolerance, seems to be related to the ability of the antigen to induce low molecular weight antibody formation. Thus marine toads could both produce positive memory

and be rendered tolerant to BSA whereas they neither respond anamnestically nor produced low molecular weight antibodies against Salmonella adelaide flagellar antigen. Xenopus laevis toads are known to produce both IgM and a low molecular weight class of antibody in response to HGG. The high dose tolerance reported in the present Chapter is, therefore, consonant with Germain and Marchalonis' findings for Bufo marinus.

- C H A P T E R S E V E N -

- C H A P T E R S E V E N -

GENERAL DISCUSSION

Animals with free-living larvae, while acquiring self-tolerance, may also encounter foreign antigens in the environment at a stage when their immune system is still extremely immature. Indeed, histogenesis of the lymphoid tissues does not occur until after hatching in many amphibian and fish species (Manning and Horton, 1969; Botham and Manning, 1981). The question of whether tolerance to xenogeneic material can be induced in these young larvae is therefore of interest. In the present work attempts were made to induce immunological tolerance during the ontogeny of Xenopus laevis using xenogeneic, non-viable cells (sheep erythrocytes, SRBC), soluble antigen (human gamma globulin, HGG) and viable allogeneic blood leucocytes administered by injection.

In some experiments the animals were injected with the antigen at an early stage of their development (Stage 48; Nieuwkoop and Faber, 1967) and in other experiments the injections were given at the later larval Stage 56 or during adulthood.

In one attempt at tolerance induction, the antigen SRBC was injected with the alkylating immunosuppressant drug, cyclophosphamide (CY). Although this method was successfully used with mice (Aisenberg and Davis, 1968; Dietrich and Dukor, 1969; Many and Schwartz, 1970) and fish (Mohan, 1977), it was found not to be as

successful in Xenopus laevis. On the contrary such treatment when attempted with adult toads led to marked augmentation of their serum haemagglutinating antibody (HA) levels and splenic rosette forming cell (RFC) response.

In chapter 5, experiments are described in which attempts were made to induce tolerance in Xenopus laevis following the well established classical method of transplantation tolerance induction which has previously been successfully used with both mammals and birds. In these previous experiments, embryonic or neonatal hosts were injected with adult live allogeneic cells and their ability to reject skin grafts from the cell donors was tested later in life. These hosts were often found to be specifically tolerant to cell donor skin allografts (Billingham et al., 1953, 1956). However, when this method was adopted for the induction of transplantation tolerance to allogeneic tissues in Xenopus laevis in the present study it was found to be ineffective. This method was unsuccessful not only for the induction of transplantation tolerance but also for priming the host for second set allograft rejection.

A further attempt to induce tolerance in Xenopus laevis was performed using a method known to be successful, not only in mammals (Golub and Weigle, 1967; Hunneyball and Stanworth, 1979) and birds (Grebenau and Thorbecke, 1978) but also in the anuran amphibian Bufo marinus (Marchalonis and Germain, 1971, 1981). In this method the antigen is usually a soluble substance which is injected at high doses into the host without adjuvant. In the experiments described in chapter 6 of the present work, HGG was used and the hosts were either

tadpoles at Stage 56 (Nieuwkoop and Faber, 1967) or adults. Such treated animals were later found to be tolerant to a subsequent challenge with the same antigen when given with adjuvant. This form of tolerance can therefore be demonstrated in adults as well as in larvae.

Following the above successful induction of tolerance to HGG an attempt was made to analyse the mechanism(s) behind the maintainance of such tolerance and at the same time to adoptively transfer the tolerant state to other animals. Spleen and thymus cells from tolerant donors (toadlets) were adoptively transferred into normal histocompatible hosts of the same age (Chapter 6). The results from this experiment showed that tolerance was not transferred, indicating that unlike similar experiments in mammals (Stocker and Nossal, 1977) and birds (Grebenau and Thorbecke, 1979), high zone tolerance to HGG in Xenopus laevis is not likely to be due to the generation of suppressor cells. However, in these experiments, as well as in the experiments described in chapter 5, more work is required to establish the effectiveness of the methods employed for the transfer of allogeneic cells. Further future experiments using a passive transfer system may reveal whether serum factors are involved in the maintainance of tolerance to HGG in Xenopus laevis, as is the case in chicken (Morgan, 1978; Morgan et al., 1978; Morgan et al., 1979) and mice (Fidler, 1979).

The present ontogenetic study appears to reveal a difference in the memory responses of Xenopus laevis to three different thymus-dependent antigens, depending on their form and viability. As in higher vertebrates, the form in which the antigen is administered into

anurans seems to affect it's "antigenicity" or "tolerogenicity". In Xenopus laevis HGG given as a solute in saline was a poor immunogen compared with when it was given with adjuvant (Manning and Turner, 1972). In the present study however, HGG was found to be tolerogenic when administered at high doses without adjuvant (Chapter 6). Similar results were recently reported to occur in Bufo marinus in response to bovine serum albumin (BSA) (Marchalonis and Germain, 1971, 1980). Alloantigens given to Xenopus laevis tadpoles in a viable cell suspension form were found not to induce tolerance in contrast to the results reported by Barlow et al. (1981) using skin grafts in the same species. In Rana both xenogeneic and allogeneic tolerance were reported to be induced when the antigen was administered in a soluble homogenate form of skin cells (Vyazove and Sorokina, 1961, 1962). Furthermore, Auerbach (1975) found that a suspension of intact foreign erythrocytes was immunogenic when injected into mice whereas a lysate of such cells was tolerogenic. Auerbach (1975) subsequently outlined a model for stem cell tolerance where the antigen forms a common determinant for the progeny of that common stem cell to which it binds. In self tolerance, the stem cell theory in essence stipulates that stem cells produce tolerogens which induce the production of serum blocking factors which in turn block the antigenic determinants on the surface of the progeny of that initial stem cell.

Experiments in the present study appear to indicate that anamnestic RFC responses to SRBC cannot be provoked in Xenopus laevis tadpoles (Chapter 3). In Rana catesbeiana, Moticka et al. (1973) reached similar conclusions when tadpoles in their experiments failed to produce secondary antibody production in response to secondary

challenge with SRBC. Moticka et al. (1973) suggested that the failure of bullfrog tadpoles to produce a true anamnestic antibody response may be due to the lack of low molecular weight immunoglobulin (LMW Ig) production in this response. Williams (1982) on the other hand, speculated that in Xenopus laevis tadpoles, the undeveloped complement system was behind this inability to detect splenic PFC in response to a hapten-erythrocyte conjugate after priming with the carrier (the erythrocyte). The possible role of a complement system in memory responses will be discussed later.

Subsequent experiments in the present study, however, have demonstrated that injected tadpoles can respond anamnestically when challenged after metamorphosis (Chapter 3 and Chapter 6). This appears to indicate that immunological memory has been provoked during the larval period. This also indicates that immunological memory in Xenopus laevis, whether positive anamnesis or tolerance, persists through the various structural changes of metamorphosis. The results of the present study therefore, support recent reports which demonstrated the persistence of memory to viable skin allografts and soluble antigens (dinitrophenyl-KLH conjugates) through metamorphosis in Xenopus laevis (Du Pasquier and Haimovich, 1976; Nagata, 1976; DiMarzo and Cohen, 1979; Manning and Botham, 1979; Botham and Manning, 1980; Cohen et al., 1980). It was surprising in the present study however, to find that the administration of viable allogeneic blood leucocytes (ABL) into Xenopus laevis tadpoles (Chapter 5) did not provoke a memory response detectable after metamorphosis.

Other investigations in the present study (Chapter 4) appear to indicate that immunological memory to injected SRBC can be induced as early as Stage 48 in Xenopus laevis. This does not necessarily entirely disagree with the report of Kidder et al. (1973). Kidder and his colleagues (1973) were unable to detect a primary RFC response to SRBC in Xenopus laevis tadpoles at Stage 48. They found that the RFC response commenced at Stage 49 in concomitance with the first appearance of small lymphocytes in the spleen. The present study, therefore, appears to suggest that memory function in Xenopus laevis develops independently and earlier than the RFC response and may be related to the lymphoid maturation of the thymus. Manning and Horton (1969) studied the histogenesis of lymphoid organs in the larva of Xenopus laevis and found that at Stage 48 only the thymus becomes lymphoid. Alternatively, the antigen may persist and stimulate the peripheral lymphoid cells as these mature.

Humoral responses to a thymus dependent antigen such as foreign erythrocytes, involve the co-operation of T and B cells. In these responses T helper cells are thought to produce antigen specific and/or non-specific factors to activate the appropriate B cell line. These factors are thought to be a type of lymphokine (Marchalonis et al. 1977; Dutton and Swain, 1982). T-B cell co-operative reactions (Edwards and Ruben, 1982) and lymphokine systems (Gearing, 1983) are known to be present in anuran amphibians (for example, Xenopus laevis). Presumably, therefore, the interactions between memory cells and effector cells in humoral anamnestic responses (for example RFC response to SRBC) may involve a kind of co-operative factor.

In contrast to the findings of Koppenheffer and Inchalik (1979)

and to the results reported in Chapter 3 for toadlets primed as larvae, the anamnestic RFC response to SRBC in adult Xenopus laevis in the present study was not significantly increased. The secondary haemagglutinating antibody (HA) response however, was noticeably higher. These observations appear to agree with those reported by Minagawa et al. (1975) in the more advanced anuran Rana catesbeiana. Minagawa et al. (1975) found the splenic PFC response of Rana catesbeiana frogs did not increase after secondary immunization with SRBC. They did, however, notice an increase in both the numbers of plasma cells and the proliferative index in the cortical regions of the thymus and the jugular body and splenic white pulp. Ruben (1975) also reported similar results obtained in his laboratory which demonstrated that in the newt, Triturus viridescens, an anamnestic splenic RFC response could not be detected after secondary immunization with horse erythrocytes (HRBC). The serum HA titre however, increased noticeably. In the same report Ruben (1975) speculated that antibody producing cells leave the spleen after secondary challenge with the antigen and continue to produce antibodies elsewhere in the immune system. By this Ruben (1975) seems to suggest that HA producing cells belong to the same cell line as that producing RFC cells. However, plaque forming cells (PFC) in the spleen can increase after secondary immunization to a greater level than the increase in serum HA. Wright and Cooper (1980) studied the anamnestic HA and splenic PFC responses to SRBC in Rana pipiens. They found that both responses increase following secondary challenge albeit the increase in the PFC response was more impressive. Plasma cells which are antibody producers can increase in the spleen in spite of the absence of PFC.

As mentioned earlier, Minagawa et al. (1975) reported that such

events took place in the spleen of adult Rana catesbeiana after secondary immunization with SRBC. Experimental results in the present study indicate that the anamnestic RFC response in adult Xenopus laevis was released from the influence of suppression, presumably brought about by suppressor cells, by treatment with cyclophosphamide (Chapter 4). Serum HA levels were also increased by treatment with this drug, suggesting that HA producing cells may have been under some suppression. Cyclophosphamide is thought to be cytotoxic to suppressor cells in mammals (Gershon, 1978; Shand, 1979).

It is possible that the direction towards tolerance in the immune response to an antigen may have some relation to the production of high levels of LWM Ig in response to that antigen. There is some evidence for this view from work done on mice (Collison et al., 1978) in their response to the hapten trinitrophenyl (TNP) conjugated onto foreign erythrocytes (RBC) as the carrier. The administration of a high dose of anti-carrier of low molecular weight class immunoglobulin (IgG) suppressed the subsequent PFC response to the hapten conjugated onto a different RBC carrier, that is the animals were tolerant to the hapten. The administration of high molecular weight class anti-carrier immunoglobulin (IgM) on the other hand had a converse effect; it led to an increase of the normal anti-hapten PFC numbers.

In Xenopus laevis it is known that the antibody response to SRBC is almost exclusively of the high molecular weight class immunoglobulin (IgM), whereas higher levels of the low molecular weight class of immunoglobulin (LMW Ig) are also produced in response to HGG. Jurd et al. (1975) have reported that LMW Ig was produced in Xenopus laevis tadpoles in response to subcutaneons implants of alum precipitated HGG

light chain. This is thought to explain to some extent the successful induction of tolerance in Xenopus laevis to HGG but not to SRBC in the present study (Chapters 6 and Chapters 3 and 4 respectively). In adult Xenopus laevis treated with CY, the augmented RFC and HA responses to SRBC reported in the present study may be due to a greater IgM production indirectly conferred by CY. It is suggested that CY may have depleted a population of cells of a suppressive nature which normally (in untreated animals) regulate the IgM producing clones.

The different influences of CY on the generation of memory when this drug is applied at different stages of development perhaps depends on the different kinetics of the response at various stages of immuno-maturation. Cyclophosphamide is known to damage those clones of cells that are metabolically active or proliferating at the time of drug administration (Bach, 1975; Shand, 1979; Internation Agency for Research on Cancer (Lyon) IARC monographs, 1981). In the present study, memory cells generated at the time of priming with SRBC may be the cell population most affected by this drug in Xenopus laevis tadpoles. In adults however, there is a possibility that suppressor cells were eliminated by CY treatment. This perhaps reflects changes in immuno-regulatory mechanisms pre- and post-metamorphosis. Moreover, it is possible that the cells affected by CY may have been T cells (thymus cells). Recent reports have presented some indirect evidence that the secondary response to SRBC in Xenopus laevis was depleted by early larval thymectomy, whereas adult thymectomy augmented the response (Gruenwald and Ruben, 1979; Ruben et al., 1980). The mechanisms by which immunoglobulins affect the memory response is not known but it may be related to the mechanisms of antigen trapping by dendritic cells

resident in various lymphoid organs, particularly the spleen of mammals (van Rooijen, 1974, 1977, 1978, 1980, 1981; Klaus et al., 1980; Mandel et al., 1980; Tew et al., 1980) birds (White et al., 1970, 1975; Hoffman-Fezer et al., 1977), reptiles (Borysenko, 1976), amphibia (Horton and Manning, 1974; Collie, 1974; Collie and Turner, 1975) and fish (Ellis, 1980; Secombes and Manning, 1980; Secombes et al., 1981; Secombes et al., 1982) (for a brief review see van Rooijen 1981). Antigen-antibody complexes are thought to be initially formed during the primary response and are then trapped in the spleen. This is thought to lead to the formation of germinal centres in the follicles of murine spleens and consequently may lead to the generation of B memory cells (Klaus et al., 1980; Kunkl and Klaus, 1981). The details of such events and their mechanisms are not clear yet, but recent reports seem to suggest that complement components (C3) may play some role in the attachment of antigen-antibody complexes to the surfaces of dendritic cells (Klaus and Humphrey, 1977; Klaus et al., 1980).

It is not certain whether such mechanisms take place in Xenopus laevis. It is known however, that the kinetics of antigen trapping in the spleens of tadpole and adult Xenopus laevis are similar to those observed in mice (Collie, 1974). Nevertheless, germinal centres as such were not observed to develop in Xenopus laevis although memory responses can still take place (Chapter 6 in the present work). It is possible that the accumulations of pyroninophilic cells at the site of antigen localization in the white pulp of Xenopus laevis toads (Horton and Manning, 1974) may be the anuran equivalent of the germinal centres of mammals and chicken. Further investigations of the histology of antigen localization in Xenopus laevis have been conducted by Baldwin and Cohen (1981) and Baldwin and Sminia (1982). They described a

dendritic type of cell associated with the sites of antigen localization in the splenic white pulps of both tadpoles and toads; they termed them XL cells. The same authors found that these cells develop by "budding off" from the central arteriole in the white pulp of spleens from larval stages between 52-56 shortly after the lymphoid differentiation of the lymphoid organ. After that they are thought to migrate towards the periphery of the white pulp within the boundary layer where they reside. In adult Xenopus laevis toads, XL cells are thought to act as a "conveyor belt" transporting antigen from the red pulp on their long pseudopods through the boundary layer into the white pulp where lymphocytes were observed to pick up the antigen (Baldwin and Cohen, 1981). What other function(s) these cells may serve is still not known. Their development however, appears to be concomitant with the factor discussed in Chapter 3 which may be required for the expression of memory.

A major finding which emerges from the present study is that Xenopus laevis tadpoles do not appear to be vulnerable to tolerance induction by xenogeneic antigens even when these are administered at an early stage in ontogeny. Tolerance in the larva was induced only by employing soluble antigen in very high doses, such as will induce tolerance in adults also. Regarded teleologically, the failure to induce tolerance to foreign materials in free-living larvae is perhaps not surprising. Nevertheless, tolerance has been reported in the humoral antibody responses of young carp fry (see Mughal, 1984). The possession of a free-living larva may necessitate a rapid maturation of the pathways which lead to positive immunity, at least for certain forms of antigenic stimulation and for certain manifestations of the immune response (see Mughal, 1984). Live allogeneic cells may be handled

differently, possibly depending on whether or not they establish a chimaeric state. Transplantation tolerance in anurans is a well documented phenomenon although it was not demonstrated under the conditions of the present experiments. The mechanisms involved when memory (tolerance or positive anamnesis) is induced in larvae and their relationship to the acquisition of self tolerance and to immunoregulation have yet to be determined.

SUMMARY

ACKNOWLEDGEMENTS

DECLARATION

SUMMARY

Xenopus laevis injected with sheep erythrocytes (SRBC) at Stage 48 of Nieuwkoop and Faber (1967) showed no evidence of tolerance induction as a result of early exposure to antigen. These experiments showed that priming during the larval stages of development (at Stages 48 and 54 or at Stage 56) led to a positive anamnestic response when the animals were challenged after metamorphosis as toadlets. It was not, however, possible to demonstrate enhanced secondary responses within the larval period itself.

The drug cyclophosphamide (an alkylating agent) had no tolerance potentiating effect when administered with a primary injection of SRBC during early larval life (Stage 48). When challenged at Stage 54 these larvae showed the same level of splenic rosette forming cells (RFCs) as those similarly immunized but not receiving cyclophosphamide. In older larvae (Stage 56) cyclophosphamide given with the primary dose of antigen abolished the secondary rise in RFC production which occurs in animals challenged as toadlets. This contrasts with the results obtained when the primary antigen injection with cyclophosphamide was administered in adult life. In adult toads, far from depressing the secondary response, the cyclophosphamide treatment actually enchanced both the number of RFCs produced on challenge and the level of secondary haemagglutinating antibody production. This difference between tadpoles and adults in the effect of cyclophosphamide on memory responses may denote differences in regulatory mechanisms. In the larva, positive memory cells generated at the time of priming may be the population most affected by the drug, whereas in the adult it is probable that suppressor cells were being eliminated by

cyclophosphamide treatment.

In contrast to their ability to react to SRBC with the induction of positive memory, larvae injected with live immunologically competent allogeneic cells (adult blood leucocytes) failed to produce any anamnestic responses when challenged as toadlets. Then cells injected by various routes into larvae from Stage 47 to Stage 57 induced neither tolerance nor positive memory. The recipients responded in a primary manner both in mixed leucocyte reactions and to skin grafts.

Toadlets treated with human gamma globulin (HGG) as Stage 54 larvae could produce good anti-HGG antibody levels after challenge. Thus, as with xenogeneic cells (SRBC), anamnesis induced in the larva was not affected by metamorphosis. The induction of high dose tolerance to HGG was demonstrated in both Stage 54 larvae and adult toads. Preliminary attempts to transfer this acquired unresponsiveness to normal toadlets using thymocytes or spleen cells taken from tolerant animals were unsuccessful .

It is concluded that, although transplantation tolerance to allografts can be induced in Xenopus laevis, the free-living larvae are not vulnerable to tolerance induction by allogeneic cells was ineffective, possibly these had failed to establish chimaerism. In the present experiments tolerance was not observed except to an antigen (HGG) injected in soluble form and at a hige dose, such as could induce tolerance in adult toads as well as in the tadpole.

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DECLARATION

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1) Johari, G.M., Botham, P.A. and Manning, M.J. (1980): "Transplantation tolerance in amphibian development". In "Aspects of Developmental and Comparative Immunology I". Ed. J.B. Solomon, Pergamon Press, Oxford, New York, Toronto, Sydney, Paris, Frankfurt, p 409-414 (Copy enclosed).

2) Manning, M.J. and Al - Johari, G.M. (1985): "Immunological memory and metamorphosis". In "Metamorphosis". Eds. M. Balls and M. Bownes, Clarendon Press, Oxford, Chapter 21 (In press).

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TRANSPLANTATION TOLERANCE IN AMPHIBIAN DEVELOPMENT

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ABSTRACT

he injection of different doses of allogeneic adult blood leucocytes intraperito-eally into Xenopus laevis larvae at various stages of development failed to induce olerance either in splenic mixed lymphocyte reactions or in skin graft rejection esponses when the hosts were tested against their donors as 2-6 month old toadlets. he results are discussed in relation to the various methods used for the induction f transplantation tolerance.

KEYWORDS

ntraperitoneal injection, Xenopus, tolerance, mixed lymphocyte reaction, llograft rejection, chimaeras.

INTRODUCTION

ransplantation tolerance may be induced by various methods. These include the oining of embryonic circulatory systems, the aggregation of embryonic blastomeres, he inoculation of cells or the application of tissue grafts during young stages of evelopment, and the reconstitution of irradiated adults with allogeneic bone arrow cells. The outcome, in terms of the operational tolerance obtained, is not ecessarily identical in all cases. The mechanisms involved may depend on such actors as dose, histocompatibility differences, the level of chimaerism produced, nd whether the histocompatible donor cells include stem cells of the immune system.

n mammals the tolerance obtained by the infusion of allogeneic cells into a new-orn receipient has been variously introduced as a clonal deletion or as involving uppressor cells or serum blocking factors. The animals are chimaeras, although hether their chimaerism is a cause or effect of the tolerant state is uncertain.

t seems that various circumstances and different levels of complete or partial olerance may lead to different sub-populations of lymphocytes becoming tolerized nd attempts have been made to analyse the condition which may lead to clonal eletion or to "active" suppression (Beverley and others, 1973; Von Boehmer, prent and Nabholz, 1975; Wright and co-workers, 1975; Brooks, 1975). For xample, using neonatal mice, Brooks found that fully tolerant animals showed no

mixed lymphocyte reactions or cytotoxic cells in vitro tests whereas partially tolerant animals often possessed cells which responded in mixed lymphocyte cultures (MLR), this effect being related to the dose of cells administered. Mammalian experiments of this nature are, of necessity, performed on relatively late stages of development (by amphibian standards) or are performed on irradiate hosts. Much earlier exposure to allogeneic tissue can be achieved in mammals by the use of allophenic (tetraparental) mice (e.g. Phillips and Wegmann, 1973). These chimaeras are created by whole embryo fusion at the 8-cell stage of development. Unfortunately, the 'dose' cannot be controlled because the 'mix' o parental type cells is not readily regulated.

Amphibians having free living larval forms, are ideally suited to studies on the early induction of transplantation tolerance. Grafts of flank tissue can be exchanged between 48 hour post-fertilization Xenopus laevis embryos resulting in animals which are tolerant of the partner's skin (Clark and Newth, 1972) and whic are unreactive against their partner when their spleen cells are reacted together in mixed lymphocyte reactions (Manning and Botham, 1979; Botham and Manning,1980 In the latter experiment cells from tolerant animals gave a heightened response with control (third party) cells from normal animals.

Xenopus laevis can, in fact, be rendered tolerant throughout the entire larval life to the antigenic product of both major and minor histocompatibility loci, th outcome depending on factors such as maturity, gene dose, and the amount of donor skin tissue grafted (DiMarzo and Cohen, 1979; Cohen, DiMarzo and Hailparn-Barlow 1980; Cohen, Hailparn-Barlow and DiMarzo, 1980).

We set out in the present experiment to investigate the relationship between the dose of allogeneic donor cells and the state of differentiation of the host, by injecting graded doses of allogeneic blood leucocytes into Xenopus larvae at various stages of development. In fact we have shown that the intraperitoneal route, although effective in mammals, is not one which elicits long term memory responses, (either positive or negative) to allogeneic cells in Xenopus larvae.

MATERIALS AND METHODS

Animals.

The animals used were obtained from the colony of Xenopus laevis in our laborator They were bred and maintained as described in Manning and Botham (1980). As larvae at different stages of development, from stage 47 to stage 57 (Nieuwkoop and Faber, 1967), the animals received single ip. injections of allogeneic leucocytes. When 2 to 6 month old toadlets, they were used for MLR's and skin grafting experiments.

Preparation and Injection of the Allogeneic Leucocytes.

Under sterile conditions, the blood was collected from the dorsalis pedis vein (see Millard, 1941) of adult leucocyte donors using heparinised pasteur pipettes. 1 ml. of blood was diluted in 1.5 ml. of heparinised Lebovitz-L15 medium (Gibco, Biocult) diluted with distilled water. This was layered on 4 mls. of lymphocyte separation medium (Flow laboratories, Irvine) in a 10 ml. centrifuge tube. The tube was centrifuged for 10-12 minutes at 800 rpm. The leucocytes, mainly consisting of lymphocytes, were collected from the cloudy layer above the separation medium. They were washed twice and resuspended in diluted L15 medium and counted. The yield of leucocytes was $9 \times 10^6 \pm 1 \times 10^6$ cells/ml of blood,

ith 1 x 10^5 ± 1 x 10^4 RBC's contamination. The leucocytes were injected ntraperitoneally into the tadpole larvae (leucocyte recipient) using no.30 gauge eedles. Control animals were injected with 2 to 10 ml. of diluted L15 medium.

n vitro Cultures (MLR).

wo-way cultures were performed by the method described in our previous ommunication (Manning and Botham, 1979). For one-way reactions, the spleen ell suspensions from the leucocyte donor and the third party control animal were ncubated for an hour at 26°C with mitomycin-C at a concentration of 0.05 mg/ml. fter incubation the cells were washed three times and resuspended at a concentration of 1 x 10^6 cells/ml. in culture medium (Manning and Botham, 1979); the method eing modified from the method developed by Bach and Voynow (1966). Mitomycin-C reatment in MLR's impairs the ability of the lymphocytes to divide (and to ncorporate thymidine) but not their stimulatory effect on the untreated cells Bach and Voynow, 1966).

he cells from the cultures were washed with distilled water on glass fiber ilters using a Flow titertek harvester. The harvested cells were placed in olythene vials to be solubilised by incubation for 1 hour at 50°C with 0.3 ml. rotosol/vial (New England Nuclear). 5 ml. of PPOP/POPOP toluene solution Scintiprep, Fisher) were added to every vial and the radioactive emissions were ounted in a liquid scintillation counter. The stimulation indices (S.I.) were alculated as the ratio of the counts per minute from the mixed cultures divided y the mean count of the control cultures.

llogeneic Skin Grafting Experiments.

xperiments were performed in a similar procedure to a previous communication Manning and Botham, 1979). Every animal to be grafted received an autograft; skin allograft from the leucocyte donor, and a skin allograft from a third party ontrol animal (see Fig. 1). The animals were replaced in standing tap water and nspected every three days for signs of graft rejection. The grafts were judged o be totally rejected when they had lost all their visible pigmentation.

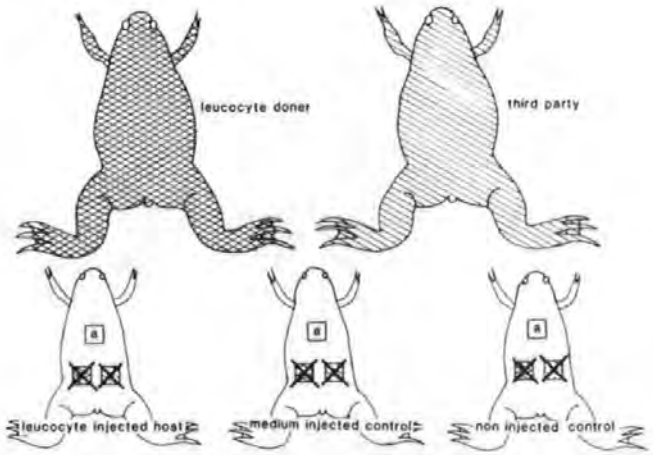

Fig. 1. Skin grafting experiment,(a)-autograft

RESULTS AND DISCUSSION

It can be seen from Fig. 2 that ip. inoculation of adult allogeneic leucocytes injected into Xenopus at stage 47, or at later larval stages, failed to induce tolerance in mixed lymphocyte reactions between host and donor spleen cells performed when the toadlets were 2-6 months old. Furthermore, third party responses between host and normal (non-donor) toadlets showed no heightened reactivity. This is in contrast to the results obtained in our earlier experiments in which transplantation tolerance was induced, at one to two days post-fertilization (Manning and Botham, 1979).

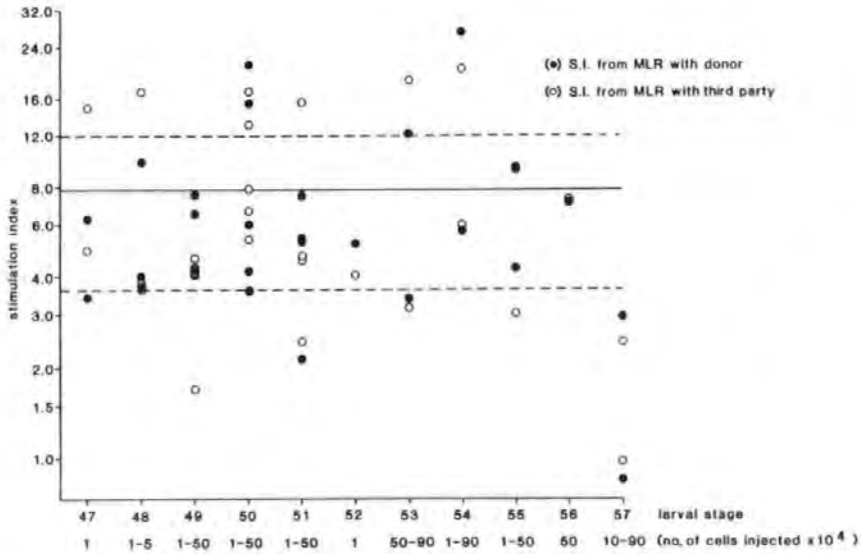

Fig. 2. S.I. from two-way MLR from experiment involving ip. injections of Xenopus larvae with allogeneic cells. (——) mean and (----) standard deviation of S.I. from normal v. normal control experiments.

Table 1 shows the results from a different group of animals from those used in the experiments shown in Fig. 2. These animals were grafted as 2 - 6 month old toadlets, with donor skin. The donor grafts were rejected at the normal rate fo a primary response, i.e. neither tolerance nor second-set immunity had been induc

Larval stage	No.of cells injected	Rejection times (days)		
		Skin grafts on injected hosts from		Normal controls
		Donor	3rd Party	
47	$1x10^4$	20, 27	13, 22	26, 26, 13
48	$5x10^4$	22, 22, 22	22, 22, 22	26, 26, 26, 26, 26, 26
51	$1x10^5$ - $5x10^5$	24, 20	24, 18	21, 21, 20, 20
53	$1x10^5$ - $5x10^5$	27, 27, 20	27, 27, 20	27, 27, 27, 27, 23, 15
54	$5x10^4$ - $5x10^5$	23, 27, 23	23, 27, 20	27, 27, 27, 27, 23, 15

Table 1. Rejection times of skin allografts in experiment involving ip. injections of Xenopus larvae with allogeneic cells.

From Table 2 it can be seen that the method of tolerance induction used in the present experiment differs markedly both from our previous experiments on 1 - 2 day old embryos (Manning and Botham, 1979) and from the experiments of Cohen and his co-workers who placed adult skin grafts onto larvae (Cohen and others, 1980). Although we did not check the level of chimaerism in our previous transplantation studies on embryos it is probable from the experiments of Tompkins and colleagues (1979) that our embryonic grafts include haemopoeitic stem cells and that the resulting Xenopus were, in fact, stem cell chimaeras. In this situation it is possible that immunocompetent cells from the donor may differentiate within the host. In these experiments on embryos we observed donor-specific tolerance accompanied by heightened third-party reactivity. The latter phenomenon (of heightened third-party reactivity) is apparently not observed, however, when tolerance is induced by grafting adult skin on to larvae (in the studies of Cohen and his co-workers).

Experiment		Result	Author
Host	Donor tissue		
embryo (Neurula/early tail bud)	orthotopic grafts of presumptive lymphoid primordia from same embryonic stage.	Tolerance with enhanced 'third-party' reactivity	Manning & Botham
various larval stages	ip. injection of mature lymphoid cells.	No tolerance (normal primary response on challenge.)	Johari
various larval stages	grafts of mature skin	Tolerance	Cohen and colleagues

Table 2. Alloimmune responses in Xenopus laevis

In our present investigation using allogeneic cells injected ip., it is possible the ip. injection did not result in long-term persistence of donor cells within the host. Our results shown in Table 1 agree with those of Hildemann and others (1958) on adult fish to the extent that these authors also obtained a primary rather than a second-set allograft rejection response following ip. priming and subsequent application of donor skin. They were, however, able to induce second-set reactivity after priming by the subcutaneous route. On the other hand the ip. route can be used to induce tolerance in neonatal mammals (Billingham and co-workers, 1957) although it is less efficient than other routes (Billingham and Brent, 1957; Billingham, Brent and Mitchison, 1957; Ohara and colleagues, 1979). It is also a route which can be used in Xenopus larvae to elicit a primary response to sheep erythrocytes (Kidder and others, 1973). The results are summarized in Table 3.

Animal	Developmental stage	Type of cells injected	Results	Author
Mouse	Neonatal	Adult allogeneic spleen cells	Tolerant	Billingham & colleagues (1953,1957) Beverley (1973)
Fish	3-4 year old adults	Whole allogeneic blood	Not tolerant Not immunized	Hildemann (1958)
Xenopus	larval stage 48	SRBC	No primary response	Kidder & colleagues (1973)
Xenopus	larval stage 49-53	SRBC	Primary immune response	Kidder & colleagues (1973)

Table 3. Effect of intraperitoneal injection of foreign cells.

It has been shown in mammals that animals tolerant to allogeneic cells are chimaeras. A possible explanation of our present results is that donor cells are not successfully transplanted using the ip. route in Xenopus. For this reason we are now setting up further experiments in which the allogeneic cells are injected into the blood stream. By testing for the persistence of donor cells together with the success or otherwise of tolerance induction, we hope to investigate any possible relationship between the level of chimaerism and the nature of the tolerant state.

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